Current Research Topics in Applied Microbiology and Microbial Biotechnology

edited by

Antonio Mendez-Vilas

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Proceedings of the II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007)

edited by

Antonio Mendez-Vilas

Formatex Research Center, Spain

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Published by

World Scientific Publishing Co. Pte. Ltd.

5 Toh Tuck Link, Singapore 596224

USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601

UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

Library of Congress Cataloging-in-Publication Data

International Conference on Environmental, Industrial, and Applied Microbiology
(2nd : 2007 : Seville, Spain)

Current research topics in applied microbiology and microbial biotechnology : proceedings of the II International Conference on Environmental, Industrial, and Applied Microbiology (BioMicroWorld2007) / Antonio Méndez-Vilas (editor).

p. ; cm.

Includes bibliographical references.

ISBN-13: 978-981-283-754-7 (hardcover : alk. paper)

ISBN-10: 981-283-754-X (hardcover : alk. paper)

1. Microbiology--Congresses. 2. Biotechnology--Congresses. I. Méndez-Vilas, A. II. Title.

[DNLM: 1. Microbiology--Congresses. 2. Biotechnology--methods--Congresses. 3. Microbiological Techniques--Congresses. QW 4 I596c 2009]

QR1.I527 2007

616.9'041--dc22

2008046073

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

Printed in Singapore.

Introduction

This book contains a compilation of papers presented at the II International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2007) held in Seville (Spain) on 28 November–1 December 2007, where over 550 researches of about 60 countries attended and presented their cutting edge research.

The main goals of this meeting were to 1) identify new approaches and research opportunities in applied microbiology presenting works which linked microbiology with research areas usually related with other scientific and engineering disciplines, 2) consolidate international collaborative efforts and 3) communicate current research priorities and progress in the field. In other words, the focus of the meeting was not only to present current science but also to discuss and synthesize research priorities and directions for the future. The contents of this book mirror this focus**.**

There were many excellent presentations in the several conference sessions and plenary lectures. The conference was structured in four main sessions covering the topics: 1) Environmental, Marine, Aquatic Microbiology/ Geomicrobiology 2) Agriculture, Soil, Forest Microbiology 3) Food Microbiology 4) Industrial Microbiology/ Future Bioindustries. And four additional special sessions dedicated to: 1) Bioremediation 2) Microbial Production of Chemicals and Pharmaceuticals/ Biosurfactants/ Biotechnologically Relevant Enzymes and Proteins 3) Medical and Pharmaceutical Microbiology 4) Techniques and Methods/ Microbiology Education.

The lectures presented by the three plenary speakers can be highlighted as good examples of modern interdisciplinary applied microbiology: Dr. J.L. Ramos Martín, from the CSIC-Spanish National Research Council (Granada, Spain), presented the lecture titled "*The toluene paradox: to eat, to tolerate or to run away*". Dr. M.L. González-Martín, from the Biosurfaces and Interfacial Phenomena Group of the Department of Applied Physics, UEX (Badajoz, Spain), expounded on "*Physico-chemistry of microbial adhesion to materials: from macroscopic surface thermodynamics to modern nano-scale technology*". Finally, Dr. Riitta Puupponen-Pimiä, from the VTT Technical Research Centre of Finland (Espoo, Finland), presented the lecture titled: "*Antimicrobial and antiadhesion properties of bioactive berry compounds*"

This book is made up of the works presented in all the above mentioned sessions. As a result of it, a complete and heterogeneous book is presented here.

We would like to thank all members of the Organizing and Scientific Advisory Committees for the work made during the conference preparation. We would also like to thank all the contributing authors for sharing their knowledge and research, as well as all the reviewers for their advice, which certainly helped to improve the quality, accuracy and relevance of the conference program and publications. Lastly, we would like to thank EVONIK INDUSTRIES, BIOLOG, AES CHEMUNEX and HORIZON SCIENTIFIC PRESS for sponsoring the conference.

We hope readers will find this book interesting and helpful to their research purposes and it helps to consolidate the conference as a regular research meeting in the field.

Antonio Mendez-Vilas
BioMicroWorld2007 General Chairman

Formatex Research Center
Zurbaran 1, 2nd floor, 1st office
06002 Badajoz
Spain

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Agriculture, Soil, Forest Microbiology

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Anti-oxidative stress enzymes in *Pleurotus ostreatus*

J. Keyhani[*,1], **E. Keyhani**[1,2], **F. Attar**[2], and **M. Hadizadeh**[2]

[1] Laboratory for Life Sciences, Saadat Abade, Sarve Sharghi 58, 19979 Tehran, Iran
[2] Institute of Biochemistry and Biophysics, University of Tehran, 13145 Tehran, Iran

[*]Corresponding author: e-mail: keyhanius2002@yahoo.com

Pleurotus ostreatus is an edible white-rot basidiomycete with medicinal and bioremediation properties. In this research, the activity of key intracellular anti-oxidative stress enzymes, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), was investigated and characterized in the mushroom. A *Pleurotus ostreatus* homogenate was centrifuged at 3,000 g for 10 min; the supernatant was centrifuged at 35,000 g for 30 min, and the new supernatant obtained, called "crude extract" was used for our studies. Results showed that up to 7.4 units SOD, 11.7 units CAT and 0.037 units POX were detectable per mg protein in *Pleurotus ostreatus* extract. All three enzymes were sensitive to KCN. Non-denaturing polyacrylamide gel electrophoresis of the extract, followed by activity staining, revealed one SOD band (estimated M.W. 44 kD), one CAT band (estimated M.W. 280 kD) and three POX bands (estimated M.W. 77, 65, and 60 kD, respectively).

Keywords catalase; peroxidase; superoxide dismutase; intracellular anti-oxidative stress enzymes; Pleurotus ostreatus

1. Introduction

Reactive oxygen species (ROS) such as H_2O_2, superoxide radical and hydroxyl radical, are normal by-products of cellular respiration and metabolism and, at low concentrations, are useful for cellular physiological functions [1]. However, their overproduction is very detrimental to cell survival. Recent advances in ROS-induced diseases demonstrated that the genetically controlled overproduction of antioxidants prevented the damages produced by drugs that generate ROS [2]. It was also suggested that the consumption of food and produce containing antioxidant products and antioxidant enzymes such as superoxide dismutase (SOD) and catalase, would be very useful in preventing health damage by ROS. The most important enzymes responsible for fighting ROS excess are SODs, catalases and peroxidases.

The oyster mushroom *Pleurotus ostreatus* is an edible white-rot basidiomycete much appreciated for its flavor as a food and for its medicinal and bioremediation properties. The medicinal benefits provided by the mushroom include hypocholesterolemic [3] and antitumor effects [4, 5] as well as improvement of the antioxidant status during ageing [6]. The bioremediation activity of the mushroom includes lignin degradation, polycyclic aromatic hydrocarbons degradation, nitrocellulose degradation and polychlorinated biphenyl (PCB) degradation [7-9]; *Pleurotus ostreatus* has also been shown to release an enzyme with aflatoxin-degradation activity [10]. These bioremediation properties result from the action of antioxidant enzymes secreted by the mushroom in the environment. Although the secreted enzymes have been extensively investigated, much less research has been conducted on the intracellular antioxidant enzymes in *Pleurotus ostreatus*, in spite of the documented beneficial effect of the mushroom on antioxidant status during ageing.

In this research, we identified and characterized the activity of key intracellular anti-oxidative stress enzymes, namely superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), in *Pleurotus ostreatus*.

2. Materials and Methods

Locally purchased *Pleurotus ostreatus* was homogenized in phosphate buffer 0.1 M, pH 7.0, then centrifuged at 3,000 g for 10 min; the pellet was discarded and the supernatant was centrifuged at 35,000 g for 30 min. The supernatant thus obtained was called "crude extract" and used for our studies.

SOD activity was measured by two different spectrophotometric methods, one based on the inhibition of pyrogallol autooxidation in alkaline solution, and the other based on the inhibition of cytochrome *c* reduction, both methods being described in reference [11]. CAT activity was assayed spectrophotometrically by following H_2O_2 dismutation at 240 nm as described in reference [12]. POX activity was measured by following spectrophotometrically the H_2O_2-mediated oxidation of o-dianisidine at 460 nm as described in reference [13], or that of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) at 414 nm as described in reference [14].

Non-denaturing gel electrophoresis of the extract was followed by activity staining for each of the enzymes tested. Electrophoresis was conducted in 10% polyacrylamide gels, either at 100 V for 5 h, or at 80 V for 20 h. Staining for SOD activity was described in reference [15]. Staining for CAT activity was performed according to reference [12], except that o-dianisidine was used as the reducing substrate; CAT was revealed as achromatic bands on a brown background. Staining for POX activity was done by incubation, for 30 min, in 50 mM citrate buffer, pH 4.5, followed by a second incubation, for 15 min, in citrate buffer 50 mM, pH 4.5, 16 mM H_2O_2, then rinsing in deionized water and incubation in 50 mM citrate buffer, pH 4.5, containing 1.9 mM o-dianisidine; POX was revealed as orange-brown bands.

3. Results

3.1 SOD activity

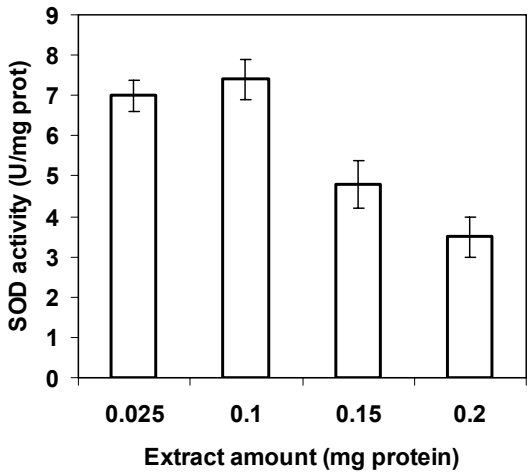

Fig. 1 SOD activity measured in various amounts of *P. ostreatus* extract. The assay was based on the inhibition of cytochrome *c* reduction. Up to 7.4 U SOD were detectable per mg of extract proteins.

In the assay based on the inhibition of cytochrome *c* reduction, SOD competes with cytochrome *c* for the flux of superoxide radical generated by the xanthine-xanthine oxidase reaction. One unit SOD was defined as that amount which caused 50% inhibition of the initial rate of cytochrome reduction. As shown in Figure 1, up to 7.4 ± 0.5 units SOD per mg extract proteins were detectable by this assay.

In the assay based on the inhibition of pyrogallol autooxidation in alkaline solution, SOD scavenges the superoxide radical that acts as a chain-propagating species in the autooxidation. One unit SOD was defined as that amount which caused 50% inhibition of the autooxidation reaction. Up to 10.4 ± 0.8 units SOD per mg extract proteins were detectable by this method, confirming the above results.

SOD activity was undetectable in the presence of 50 mM KCN.

A single band was detectable after electrophoresis of *P. ostreatus* extract proteins in polyacrylamide gel, under non-denaturing conditions, followed by activity staining for SOD, indicating the presence of at least one SOD isoenzyme, with an estimated molecular weight of 44 kD, in the extract (Fig. 2).

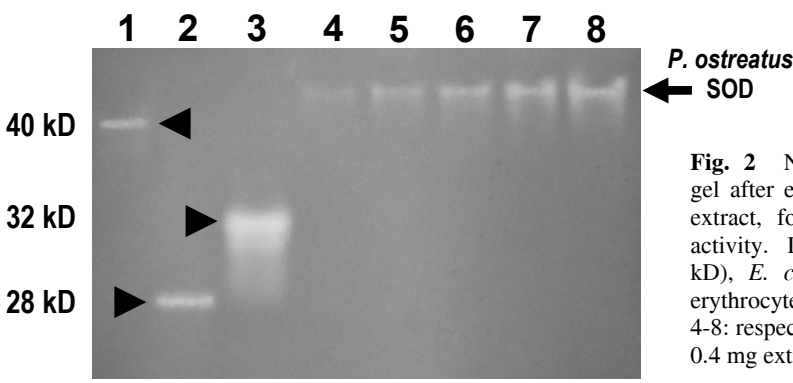

Fig. 2 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for SOD activity. Lane 1-3: *E. coli* Mn-SOD (40 kD), *E. coli* Fe-SOD (28 kD), human erythrocytes Cu/Zn-SOD (32 kD); lanes 4-8: respectively, 0.05, 0.1, 0.15, 0.25 and 0.4 mg extract proteins.

3.2 CAT activity

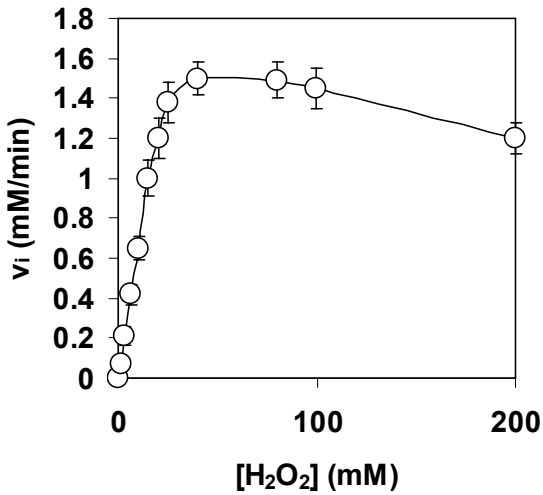

Fig. 3 Effect of H_2O_2 concentration on CAT activity in *P. ostreatus* extract. The hyperbolic plot is characteristic of Michaelis-Menten kinetics. Substrate inhibition was observed for H_2O_2 concentrations above 80 mM.

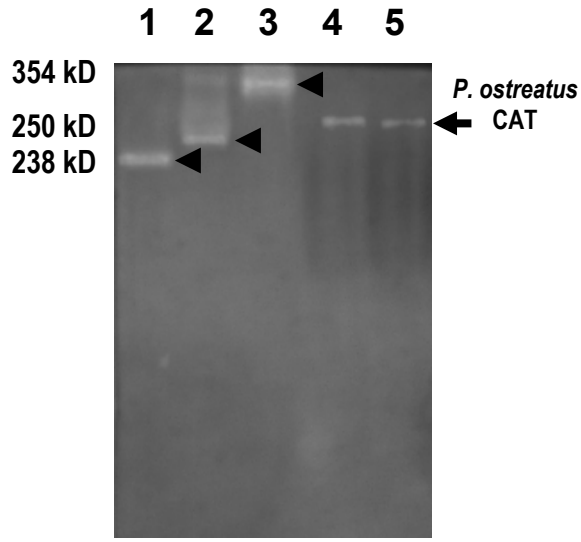

Fig. 4 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for CAT activity. Lanes 1-3: Guinea pig liver CAT (238 kD), Beef liver CAT (250 kD), *A. niger* CAT (354 kD); lanes 4-5: 0.4 and 0.3 mg extract proteins.

When *P. ostreatus* extract was added to a reaction mixture containing phosphate buffer 0.1 M, pH 7.0 and H_2O_2, the absorbance at 240 nm of the mixture would decrease steadily, indicating H_2O_2 dismutation and the presence of catalase activity in the extract. The enzymatic activity increased as a function of increasing substrate concentration, reaching a maximum value at 40 mM H_2O_2 (Fig. 3). Substrate inhibition was observed for H_2O_2 concentrations above 80 mM (Fig. 3). The maximum activity detected corresponded to 11.7 ± 0.7 units CAT per mg extract proteins (one unit CAT decomposes 1 μmol H_2O_2/min). The enzymatic activity was inhibited by KCN with an IC_{50} of 4 mM.

When the extract was electrophoresed in non-denaturing polyacrylamide gel that was subsequently stained for CAT activity, one band was detectable (Fig. 4), indicating the presence of at least one CAT isoenzyme, with an estimated molecular weight of 280 kD, in the extract.

3.3 POX activity

P. ostreatus extract catalyzed the H_2O_2-mediated oxidation of o-dianisidine and ABTS, two of the substrates used by peroxidases. Typical Michaelis-Menten plots were obtained for the variation in reaction velocity as a function of either o-dianisidine (Fig. 5a) or ABTS (Fig. 5b) concentration. The apparent K_m and V_{max} values deduced from the plots were, respectively, 0.06 ± 0.004 mM and 9.4 ± 0.4 μM/min for o-dianisidine as the varied substrate, and 0.6 ± 0.03 mM and 2 ± 0.05 μM /min for ABTS as the varied substrate.

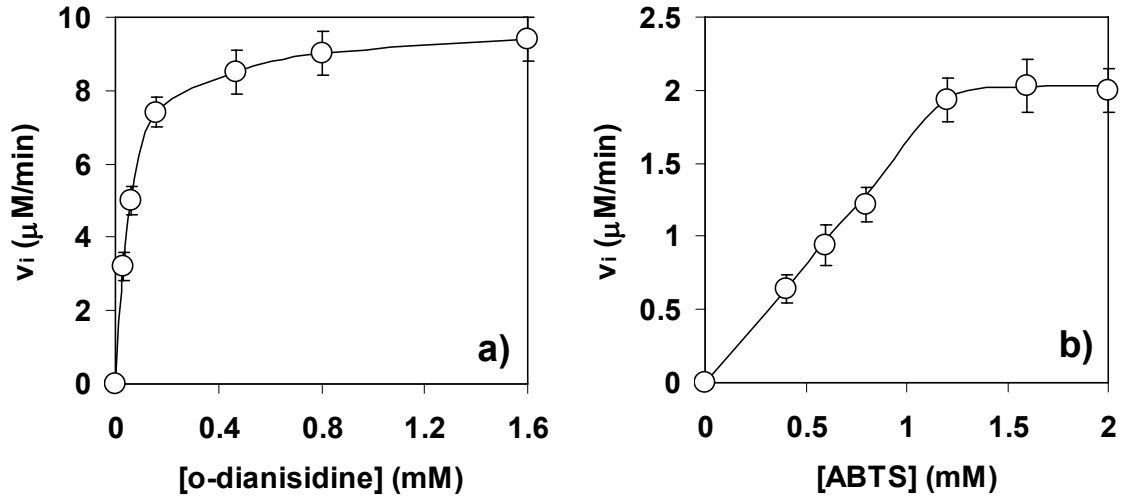

Fig. 5 Effect of o-dianisidine (a) and ABTS (b) concentration on POX activity in *P. ostreatus* extract. The hyperbolic plots are characteristic of Michaelis-Menten kinetics.

The maximum activity detected corresponded to 0.037 ± 0.002 units POX per mg extract proteins when o-dianisidine was used as the reducing substrate, and to 0.032 ± 0.003 units POX per mg extract proteins when ABTS was used as the reducing substrate (one unit POX oxidizes 1 μmol substrate/min).

P. ostreatus peroxidase activity was inhibited by KCN with IC_{50} of 0.1 mM when o-dianisidine was the reducing substrate and 4 mM when ABTS was the reducing substrate.

Electrophoresis of *P. ostreatus* extract in non-denaturing polyacrylamide gel followed by staining for POX activity revealed three bands with estimated molecular weight of 77 kD, 65 kD and 60 Kd, respectively (Fig. 6).

4. Discussion

The medicinal properties of edible mushrooms are now under intense investigation. Among the edible mushrooms, *P. ostreatus* has been recognized not only for its beneficial effects on health, but also for its bioremediation properties that have been extensively studied. Various *P. ostreatus* extracts have been found to have distinct medicinal properties [3-6] and even DNA isolated from *P. ostreatus* has been reported to have biotherapeutical potential in mice with solid Ehrlich carcinoma [16]. Although antioxidant properties of the mushroom have been reported, and a number of antioxidant enzymes secreted by the mushroom have been extensively studied, little is known on the intracellular antioxidant enzymes in *P. ostreatus*.

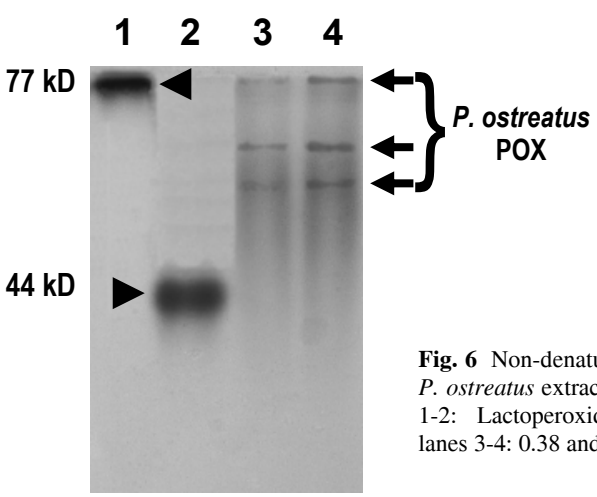

Fig. 6 Non-denaturing polyacrylamide gel after electrophoresis of *P. ostreatus* extract, followed by staining for POX activity. Lanes 1-2: Lactoperoxidase (77 kD), Horseradish peroxidase (44 kD); lanes 3-4: 0.38 and 0.5 mg extract proteins.

In this work, SOD, CAT and POX activities were detected in *P. ostreatus* extract. At least one isoenzyme of SOD with an estimated molecular weight of 44 kD was present; its sensitivity to KCN indicated that it was a Cu/Zn SOD [15]. The SOD activity of the extract, expressed as U/mg protein, was comparable to that found in

extract obtained form dormant saffron corm [11], but roughly 5 times less than that found in *Satureja hortensis* roots [15]. At least one CAT isoenzyme of estimated molecular weight 280 kD was identified; the activity was about half that found in dormant saffron corm extract [12]. As far as POX is concerned, *P. ostreatus* exhibited an activity that was 10 times less than that found in dormant saffron corm using o-dianisidine as the reducing substrate but was about twice that found in dormant saffron corm using ABTS as the reducing substrate [13, 14]. Due to their ability to use a broad range of substrates, numerous peroxidase isoenzymes have been found, particularly in plants. As it appears in this work, at least three POX isoenzymes with estimated molecular weights of 77 kD, 65 kD and 60 kD, respectively, were detectable in *P. ostreatus* extract.

Acknowledgements Supported by the J. and E. Research Foundation, Tehran, Iran.

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Beauveria bassiana mutants display a different protein profile in comparison with parental strain

A.G., Alcázar-Pizaña[1], A. Flores-Soto[1],V.E., Aguirre-Arzola[1], K. Arevalo-Niño[1], H. Medrano-Roldán[2], M. Iracheta-Cárdenas[1] and B. Pereyra-Alférez[1,*]

[1] Instituto de Biotecnología. Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León. Pedro de Alba y Manuel L. Barragán S/N. Cd. Universitaria. San Nicolás de los Garza, NL. 66450. México
[2] Instituto tecnológico de Durango. Durango, Dgo.

[*]E. mail: bpereyra@fcb.uanl.mx

The mode of action of entomopathogens has revealed that a number of extracellular enzymes, toxins and pigments are involved in pathogenesis. *Beauveria bassiana* strain BbCh1 was mutagenized using UV light. About 200 colonies were obtained after UV exposure, several colonies showed a large and stable protein hydrolysis halo. Protein content was similar for mutant and parental strains. General enzymatic activity against azocasein was higher (at least two times) in mutant strains.

Keywords *Beauveria bassiana*, proteases

1. Introduction

Natural occurring entomopathogens are important regulatory factors in insect populations, where fungus and bacteria play an important role as agents for biological control of insect pests. The entomopathogenic fungus *Beauveria bassiana* is one of the most used biological control agent, because this fungus infects a wide range of hosts. As all insect pathogenic fungi, *B. bassiana*, invade the host through the exoskeleton and the mode of entry is considered to be a combination of mechanical pressure and enzymatic degradation. Infection by *B. bassiana* is initiated when the conidia contact with the cuticle of a susceptible host and adhere by hydrophobic mechanisms [1]. During fungal penetration, the cuticular components (chitin fibrils embedded in a protein matrix) must be hydrolyzed; this fact may be done by number of extracellular enzymes, mainly by proteases, lipases and chitinases [2]. It is believed that extracellular proteases play a key role in cuticle hydrolysis and fungal entry to the hemocoel, since proteins are the major components of insect cuticle [3]. Several proteases produced by *B. bassiana*, BBP bassiasin I and Pr1, have been previously reported [4, 5]. and recently, genes encoding these enzymes have been isolated and analyzed [5].In this study, we report the mutagenesis and the isolation of several mutant strains of *Beauveria bassiana* showing higher protease activity and displaying different protease-profile than parental strain.

2. Materials and methods

Strains and culture conditions
Beauveria bassiana strain BbCh1 was maintained on yeast/peptone/dextrose/agar (YPDA). YPDA contained yeast extract (3.0 gl-1); peptone (10gl-1); glucose (20.0 gl-1) and agar-agar (20.0 gl-1). Sporulated cultures were overlaid with mineral oil and stored at 4ºC.

Mutagenesis and plate screening
Samples of four milliliters of a conidial suspension ($1X10^6$ spores/ml) were placed in a small Petri dish. The spore suspension was exposed to UV light (254 nm) for 0, 2, 4, 6, 8 and 10 min at a distance of 10 cm. The mutagenized spores were plated onto agar medium supplemented with skimmed milk (CA) as reported [6] and incubated for 7 days at 28ºC in darkness. Mutant strains were screened for their ability to produce larger zones around the colony. In order to test stability of mutant strains, isolated colonies showing larger clearing zones were repeatedly subcultured onto YPDA and CA.

Induction of protease activity
B. bassiana parental and mutant strains (10^7 spores) were inoculated in 500 ml Erlenmeyer flasks containing 100 ml of yeast/peptone/dextrose broth (YPD). Flasks were incubated on a orbital shaker at 180 rpm and 28ºC for 3 days. The culture contents were filtered trough a 0.45 μm pore size membrane, and the mycelium derived from 20 ml of dextrose sabouraud culture was washed twice with sterile distilled water, and filtered again trough the same filter. The mycelium resulting, was used to inoculate 100 ml of a subsequent growth medium MMG,

this medium consisted of 1% (w/v) gelatin in a basal salt medium (pH 7.0) composed of NaCl, K_2HPO_4 and $MgSO_4.7H_2O$ (0.3 gl^{-1} for each) and incubated at the same conditions. After 4 days of growth, the culture contents were filtered trough a 0.45 μm pore size filter and the supernatants were lyophilized and stored at –20ºC.

Determination of proteolytic activity

For the assay of general proteolytic activity, azocasein was used as a substrate [7]. Briefly, 120μl of a suitable dilution of enzyme solution was added to 480 μl of azocasein (1% w/v in 25mM Tris-HCl buffer, pH 7.6, containing $MgCl_2$ (final concentration 5 mM), and the mixture was incubated at 30ºC for 30 min. The reaction was finished by adding 600 μl of 10% (w/v) trichloroacetic acid and left for 30 min on ice, followed by centrifugation at 15,000xg at 4ºC for 10 min. Eight hundred microliters of supernatant were neutralized by adding 200 μl of 1.8N NaOH, and the absorbance was measured at 420 nm. One unit of enzyme activity was defined as the amount, which yielded an increase in A_{420} of 0.01 in 30 min at 30ºC.

Protein determination

Total protein was measured by the Bradford method [8] using Bovine Serum Albumin (BSA) as a standard.

Zymograms

To assess the protease profile of parental and mutant strains, 8.0% polyacrylamide gels were used for casein zymograms. The samples were loaded into the gel, and electrophoresis was performed at 4ºC. The gel was soaked in a 1% (wt/vol) casein solution in 0.1 M Tris buffer for two hours at 4ºC. After, the gel was washed twice with distilled water and with 0.1M Tris buffer, and then incubated for 2 h at 30ºC. The protease activity (clear zones) was reveled staining the gels with Coomassie brilliant blue.

Activity dots

Electrophoresis was performed at 4ºC and transfer was carried out in a Hoefer Scientific Instruments Transphor Electrophoresis Unit mod TE22, with transfer buffer Tris/Glycine/Methanol/water (3/14.49/200/800) as recommended by fabricant. Activity dots against specific synthetic substrates N-a-Benzoyl-DL-Arginine-r-Nitroanilide (BApNA) (trypsin like amidase activity), N-Succinyl-Ala-Ala-Pro-Leu-r-Nitroanilide (SA2PLrNA) (elastase like activity), N-Succinyl-Ala-Ala-Pro-Phe-r-Nitroanilide (SA2PFrNA) (Chimotrypsin like activity), were done to determine the type of activity of each protease [9].

Enzymatic inhibition on activity dots

Before electrophoresis in polyacrylamide 8% gels, the samples were incubated with PMSF a serino proteases inhibitor (5M final concentration), for 15 min. After electrophoresis the gel was transferred to a nitrocellulose membrane, previously saturated in the substrate (N-Succinyl-Ala-Ala-Pro-Phe-r-Nitroanilide, Chymotrypsin like activity). The membrane was incubated then at 30ºC and finally the activity was observed.

Induction-Repression of protease production

Growth kinetics in different culture media (Inducer culture media, repressor culture media, non-inductor culture media and YPD), were performed in order to determine if the regulation of protease production was affected by mutagenesis, and the activity was monitored by the use of zymograms.

3. Results

Mutant development and stability of mutant strain

Placing UV lamp at 10 cm from conidial suspension and irradiating it for 4 min, it was obtained less than 1% of surviving (Fig 1). After exposure to UV, it was obtained about 10000 mutant colonies. These colonies were screened for their ability to produce larger clearing zones around the colony when were grown on agar medium plus skimmed milk for seven days.

To asses the stability of mutant strains, they were subcultured onto YPDA and CA. The five mutant strains selected were stables for at least five repeated alternate subcultures.

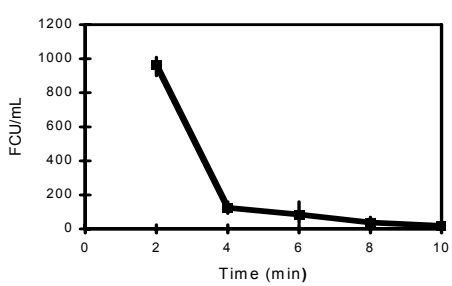

Fig. 1 Surviving percentage of *B. bassiana* strain BbCh1 after mutagenesis.

In Table 1 we present the size of hydrolysis halo of the different strains throughout five subculturing onto CA, The hydrolysis halo of mutant strains is bigger than parental one. Five mutant strains designed as M-7, M-24, M-36, M-41 and M-82 presented the largest clearing zones around the colony at seven days of growth on CA

medium. Colony morphology of mutant strains was similar to parental strain, with white to cream colonies and abundant sporulation. Mutant strains 41 and 82, presented limited growth and mutant strain M82 presented poor sporulation, but this strains develop one of the largest hydrolysis halo on CA.

Table 1: Hydrolysis halo size (mm) of parental and mutant strains

Strain	SC 1	SC2	SC3	SC4	SC5
PS	2.5	2.5	3.0	3.0	3.0
M-7	4.0	4.0	4.0	4.0	4.0
M-24	4.0	4.0	4.0	4.0	4.0
M-25	4.0	3.5	4.5	4.0	4.5
M-36	3.0	3.5	4.0	4.0	4.0
M-41	4.0	4.0	4.0	4.0	4.0
M-82	4.0	4.5	4.5	4.5	4.5

PS=Parental strain; SC= Subculturing

Induction of protease activity

General protease activity and total content of protein were measured daily. The highest content of protein was obtained at third day, all strains show similar content of protein than wild type strain except M-41 and M-24, that presented lower content of protein. When *B. bassiana* was grown on salts medium with 1.0% gelatin, the maximum protease activity was detected at third day (72 h), with increased activity (at least 2 times) in all the mutant strains.

Protease profile

A casein zymogram was used to assess the number of extracellular proteases produced by the different strains. It revealed different protease profiles in some mutant strains as M7, M36 and M82 (Fig 2 panel a), all of them showed a principal band of proteolytic activity of molecular weight about 40kD.

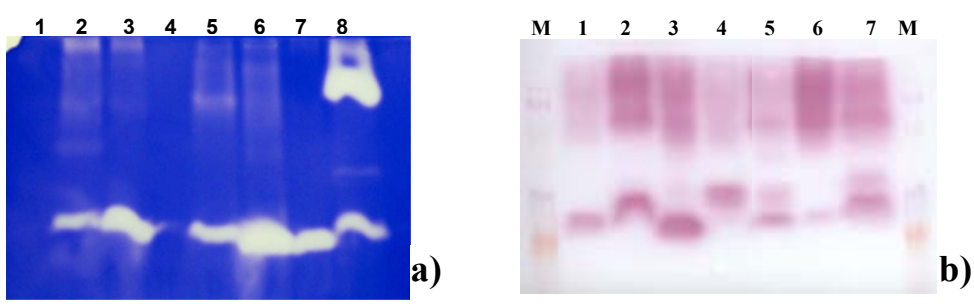

Fig. 2 Protease profile of parental and mutant strains (panel a), and activity dots against synthetic substrate for chimotrypsin like activity (SAAPPpNA(panel b); **a) 1,** Parental strain; **2,** M-7; **3,** M-24; **4,** Negative control; **5,** M-25; **6,** M-36; **7,** M-41; **8,** M-82. **b) 1,** Parental strain; **2,** M-7; **3,** M-24; **4,** M-25; **5,** M-36; **6,** M-41; **7,** M-82, **M** Molecular weight markers.

As it has been referred some of the mutant strains showed more than one protease, therefore, activity dots against specific synthetic substrates were done to determine the type of activity of each protease. None of the proteases of mutant strains showed trypsin like activity, but all of them showed chymotrypsin and elastase like activities (Fig 2 panel b).

Activity dots against Synthetic substrates

All proteases produced by the different strains were tested for inhibition by phenylmethylsulfonyl fluoride (PMSF), the results showed an inhibition of the activity of all the proteases produced by all strains, indicating that they are serin proteases.

Studies of induction-repression of protease production

No enzymatic activity was detected in parental and mutant (M-82) strains when they were growth in YPD medium or repressor medium. When they were growth in inductor medium, the protease activity was detected at 48, 72 (maximum detection) and 96 h for parental strain and 24, 48 (maximum detection) and 72 h for mutant strain M-82.

3. Discussion

In the past years many of the research on entomopathogenic fungi was oriented to the better understanding of the mode of action and to elucidate the precise role of proteases in insect infection process. The development of protease deficient strains of *B. bassiana* using UV light, has been an important tool to assess the role of proteases in pathogenicity. On the other hand it is a good tool to generate improved strains of entomopathogenic fungi [10,11] and correlated it with virulence. In this study we obtained stable mutant strain overproducers of protease obtained by UV mutagenesis.

Over producers protease selection was done by means of skimmed milk proteins degradation on plate, measured by clearing zones around the colony. This is a good screening method to select over producing strains, since it allow us a rapid examination of large amounts of colonies after mutagenesis and to evaluate in a indirect way protease production. *Metarhizium anisopliae* produces a similar range of proteases in cultures with skimmed milk and with insect cuticle [6].

In this study, we could isolate five strains showing largest clear hydrolysis halo than parental strain. The mutants M-7, M-36 and M-82 displayed different proteases profile. Mutants M-24 and M-41 have same profile but with increased activity than parental strain. Some strains show a different protease profile, but it doesn't mean that all of them are toxic against insects. The differences in the protease number produced by each strain could be since UV mutation is random and some other genes that codifie to proteases can be deregulated**.** The proteases reported produced by this fungi are proteases with a molecular weight of 36 kDa and they termed toxic against insect [4,5]. Serine proteases are widely distributed among fungi and are almost entirely extracellular, their molecular weights are commonly in the range of 18 500 to 35 000 KDa and they are optimally active at alkaline pH [12]. According to activity dots, proteases of parental strain as well as mutant strains showed chymotrypsin like and elastase like activities, the proteases showed no trypsin like activity, on the basis of no amide-hydrolyzing activity of BAPNA, this is in accordance with other purified proteases of *B. bassiana* [13,14,5], the proteases produced by parental strain and mutants were inhibited by PMSF. Strains with an increased protease activity could be considered as good tools for biological control of insect pests, since it had been demonstrated that proteases have the dominant role during cuticular penetration events.

Acknowledgements This work was supported by a PAICYT grant CN908-04. A. Alcázar-Pizaña was a recipient of a fellow from the CONAYT

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Culturable microbial populations in a vineyard soil under different management regimes: Influence on spontaneous must fermentation

A. Muñoz González[*,1], A. López Piñeiro[2], and M. Ramírez Fernández[1]

[1] University of Extremadura, Area of Microbiology. Avda Elvas s/n, 06071 Badajoz, Spain.
[2] University of Extremadura, Area of Edafology.

[*]Corresponding author: e-mail: anamg@unex.es , Phone: +34 924289426

Vineyard ecosystems involve complex interactions between plants, soil, grapes, and their accompanying microorganisms, mainly yeasts, which are closely linked to wine production. The aim of this study was to analyze the effect of vineyard management on these ecosystem interactions. Soil and must-fermentation microbial populations were analyzed over the course of a year for two management regimes: conservation agriculture (with natural cover crop), and traditional tillage. Soil and must-fermentation microorganisms were found to be correlated, and the management regime determined significant differences in the soil microbial populations. There were marked changes in yeast populations throughout the year, with a major increase in the soils at harvest under both management regimes.

Keywords vineyard ecosystem; spontaneous fermentation; soil microorganisms; ecological agriculture; wine yeast.

1. Introduction

The vineyard is a very traditional culture in the Mediterranean area. Traditional winemaking is still done by spontaneous must fermentation in most wine producing zones, although modern technologies, that use selected commercial yeasts for must inoculation, are widely spreading for a rather standard industrial winemaking. Generally, vineyards are on degraded or poor soils with poor biochemical properties [1]. Also, the conventional viticulture is considered to be a very aggressive management for the soil, because of the frequent tillage and pesticide use. An interesting alternative is to use Conservation Agriculture management, as is being used in many places all over the world, which protects the vineyard soil from wind and water erosion by using a vegetable cover and avoiding tillage [2].

The aim of this study was to analyze the effect of Conservation Agriculture on microorganisms associated with the vineyard ecosystem, by comparing with Conventional Tillage management. For this purpose, soil and fermentation microorganism populations were studied during the vineyard culture cycle.

2. Material and Methods

2.1 Soil sampling

The study vineyards are located close to Badajoz (South-West Spain), in a "semiarid Mediterranean" zone (UNESCO climatic classification). Samples were taken from two contiguous 7-year-old vineyards with *Tempranillo* grape variety. One of them is managed with Conservation Agriculture (CA) by using spontaneous vegetation cover all the year, and avoiding the use of herbicides by means of mechanical mowing. The other is managed with traditional Conventional Tillage (CT).

2.2 Microbiological analysis

Duplicate soil samples were taken from the surface layer (0-10 cm depth) of each soil in different stages of the vineyard culture during one year. At this depth, microbial development and activity is the greatest [3]. Samples were passed through a 2 mm sterile sieve, resuspended in sterile water (5 g 50 ml^{-1}), and diluted from 10^4 to 10^6 times with sterile distilled water. To obtain the greatest possible amount of culturable microorganisms [6], 100 µl aliquots were spread onto different agar culture media: YEPD (Yeast Extract Peptone Dextrose), RB (Rose Bengal agar with chloramphenicol), peptone, TSA (Trypticase Soy Agar), green malachite, special medium for *Azotobacter* and *Azomonas*, starch-casein, potato-dextrose with tartaric acid, and Sabouraud with chloramphenicol [4,5]. Total culturable microorganisms for each sample, taken as the sum of the means of duplicate subsamples for each culture medium, were expressed as total colony forming units in dry soil,

CFU g^{-1}. The plates were incubated at 25 °C up to 10 days to detect slow-growth microorganisms. As many as 32 bacterial and yeast morphological types were detected by analyzing colony morphology, cell shape (microscopy), sporulation capability, and Gram staining. Bacteria and yeast species were identified by 16-18S DNA sequencing after DNA extraction and purification from the isolated colonies [7].

Sterile must (*Macabeo variety*, pH 3.3) fermentations were performed with soil inocula ($3 \cdot 10^{-2}$ g/mL) from each sample.

Spontaneous must fermentations were done in the laboratory with the harvested *Tempranillo* grapes from each vineyard.

3. Results

3.1 Bacteria populations in soils

The CA and CT vineyards had different culturable bacterial populations (Fig. 1). The CA soil had a greater amount of culturable bacteria and a higher biodiversity than CT soil. Similar results have been found previously for different crops [8,9].

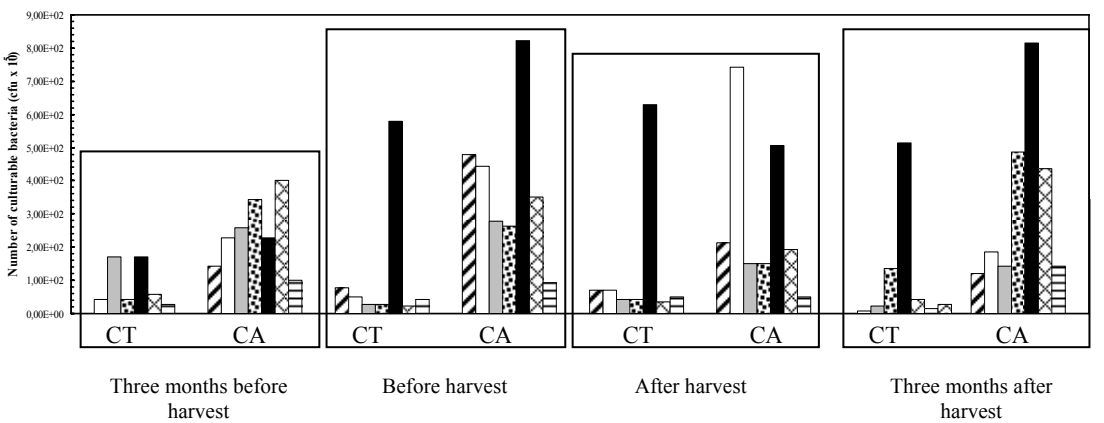

Fig. 1 *Major groups of culturable bacteria in each soil during a year.* ◪: *Stenotrophomonas;* □: *Bacillus;* ■: *Streptomyces;* ■: *Arthrobacter;* ⊠: *Pseudomonas;* ⊟: *Rodococcus;* ⊠: *Janthinobacterium.*

3.2 Yeast populations in soils

The culturable soil yeasts increased during the grape ripening, by up to more than 800% just before or after the harvest with respect to other sampling times during the year (Fig. 2). This increase should be because of the presence of fermentable sugars in the vineyard from ripened grapes, which increases the fermentative yeast population. However, the differences in soil culturable yeasts were also due to the soil management type. The CA soil had a greater amount (24%) of yeasts than the CT at harvest time, and this difference increased to up to 55% after harvest.

The yeast colonies appeared very rapidly in the soil-seeded agar media plates (24-48 hours), which indicates that yeast from these soils are in a vegetative stage rather than as spores, what would take longer time for colony appearance.

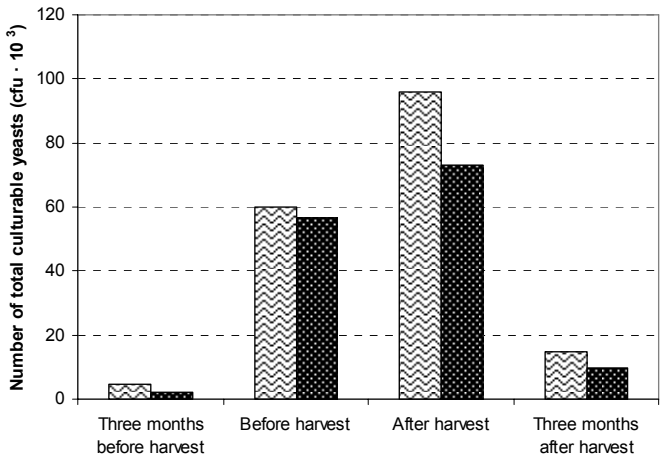

Fig. 2 *Amount of culturable yeasts in both soil managements during the year*. ☑: CA management; ▩: CT management

3.3 Must fermentations

3.3.1 Spontaneous must fermentation

The kinetics of spontaneous must fermentation was a little slower in the CT- than in the CA-vineyard must (Fig. 3). The apiculate yeasts were predominant in tumultuous and end fermentation stages of CT must, which could explain the slowness of this fermentation; while *Saccharomyces* yeasts predominated in the CA must fermentation at the same stages.

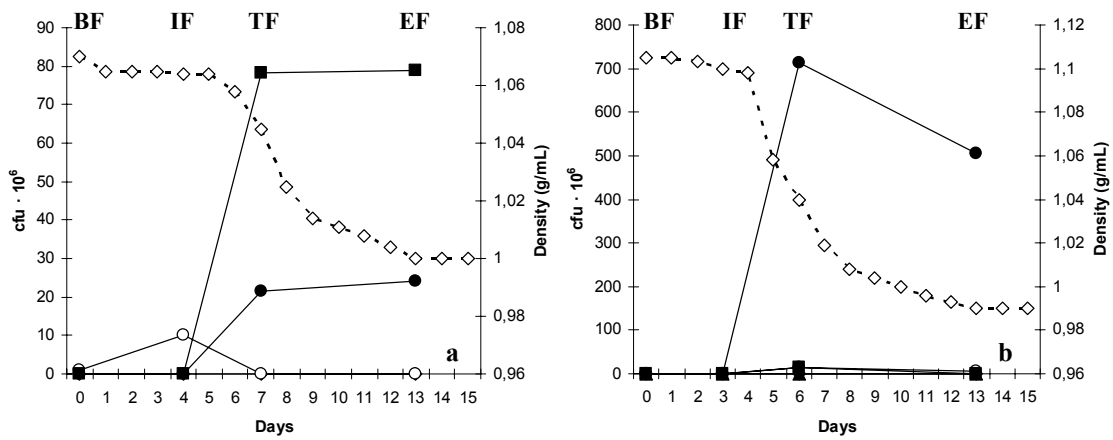

Fig. 3 *Fermentation kinetics and amount of viable microorganisms in spontaneous fermentations***.** a: fermentation of grapes from the CT-vineyard; b: fermentation of grapes from the CA-vineyard.. ▲: bacteria; ●: Saccharomyces; ○: No-Saccharomyces ■: apiculates; -◊-: must density. BF: before fermentation; IF: initial stage of fermentation; TF: tumultuous fermentation; and EF: end of the fermentation.

3.3.2 Fermentation of sterile must inoculated with soil samples

Fermentative yeasts were found in all inoculated soil samples, as soil inoculation led to fermentation onset, and sometimes even complete fermentation to dry wine, while the un-inoculated control remained unfermented. Notable differences were found depending on the time of sampling, and the soil management (Fig. 4). The must inoculated with all the CA soil samples was always finished, but the started fermentation stopped or became sluggish when the must was inoculated with most CT soil samples. The only CT-soil sample that led to complete must fermentation belonged to harvest time sampling, and it had the greatest amount of yeasts of all the CT-soil samples (Fig 2). In sum, the CA-vineyard soil contained more appropriate must fermentative yeasts than that of the CT-vineyard.

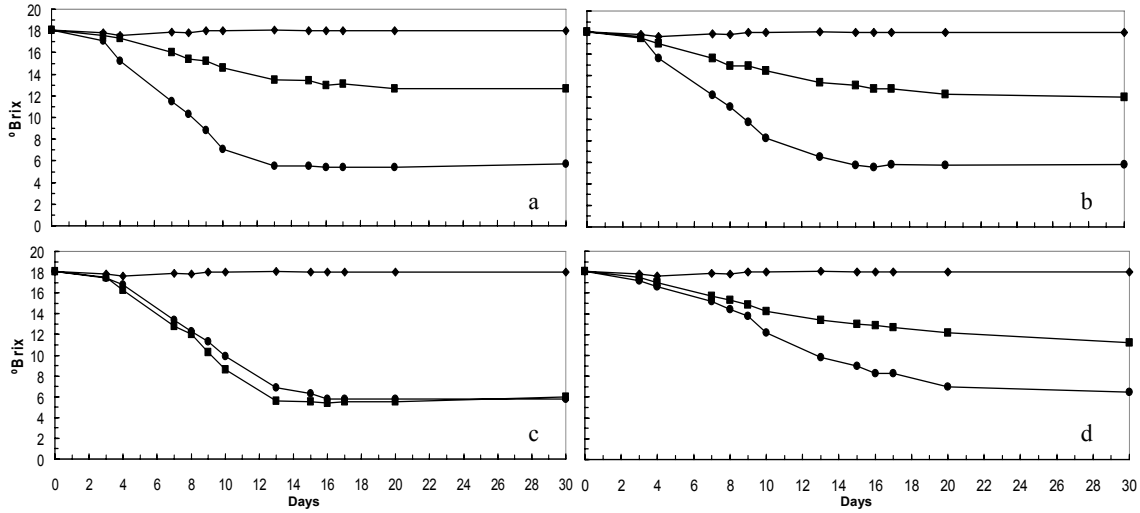

Fig. 4 *Fermentation kinetics of sterile must inoculated with soil samples.* a: Soil sample obtained three months before harvest; b: just before harvest; c: just after harvest, and d: three months after harvest. ●: CA vineyard; ■: CT vineyard; □: Non-inoculated sterile must (control).

As was the case with the bacteria (Fig. 1) and the non-fermentative yeasts (Fig. 2), the CA soil seemed a better reservoir for fermentative yeasts than the CT soil. This could be because the CA vegetation cover protects soil microorganisms from the stressing changing weather conditions, while the tillage of CT management increases the effect of external weather impacts on soil microorganisms.

4. Conclusions

The CA-vineyard soil contained higher amounts of culturable bacteria and yeasts than the CT-vineyard soil. Despite this, there was a marked increase of fermentative yeasts close to harvest time in both vineyard soils, when the grapes ripen. Nevertheless, fermentative yeasts were also found in other periods throughout the year in the CA-vineyard soil. There was a correlation between the amount of yeast in the soil and the capacity of the inoculated soil sample to initiate and complete sterile must fermentation. This raises the possibility that the vineyard soil could be a reservoir for fermentative yeasts during the inter-vintage periods, and the place of origin of the fermenting yeasts responsible for the earliest spontaneous must fermentation of each vintage.

Acknowledgements The support by the "Junta de Extremadura" to the project 2PR04B003 and the research fellowship of the main author is gratefully acknowledged.

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Diagnosis and association of *Olpidium bornovanus* and MNSV with vine decline of melon in Honduras

M. de Cara[1], D. Palmero[2], M. Santos[1], M.C. Córdoba[3], C. Jordá[3], J.C. Tello[1]

[1] Dpto. Producción Vegetal. Universidad de Almería. Spain.
[2] Dpto. Producción Vegetal: Botánica y Protección Vegetal. E.U.I.T. Agrícola. Universidad Politécnica de Madrid. Spain.
[3] Dpto. Ciencias Agroforestales. University of Valencia. Spain.

This work was supported in part by a grant (AP20033703) from the FPU program of the Ministerio de Educación y Ciencia de España.

Thirty soil samples, from 6 different farms with vine decline symptoms, plus two peat samples from a nursery were collapsed plants were previously sown, were analyzed for presence of organisms associated with the disease. With a soil-dilution plating method, only Macrophomina phaseolina and Acremonium were detected, in 2 samples each fungus. With a melon bait plant technique, named "soil phytopathometry", Olpidium bornovanus often together with Melon necrotic spot virus, was found in 70% of all the samples, corresponding with all the farms studied and the peat. Other pathogens that were detected less frequently included Monosporascus cannonballus (3.3%) and Rhizoctonia solani (3.3%). No Plectosporium tabacinum neither Rhizopycnis vagum (other two fungi associated with vine decline) were detected. Fusarium solani, that was detected very frequently (87%), was not associated with disease occurrence after a pathogenicity test. Consequently, O. bornovanus and MNSV were uniquely associated with disease occurrence and thus are the most probable cause of melon vine decline in the fields studied.

Additional keywords Collapse, sudden wilt, methyl bromide.

1. Introduction

Approximately 9,000 ha of muskmelons, mainly cantaloupe and honey dew types, are grown annually in Honduras, mostly for export to the USA. Melon is the most important horticultural crop in the provinces of Choluteca and Ojo de Agua in Honduras. Traditionally, three sixty-day-cycles of melons are grown annually beginning in September and ending in April. Each year farmers disinfest the soils with methyl bromide or other chemicals to control soilborne diseases and weeds. In recent years, the most important soilborne disease in melon is a vine decline disease, which symptoms are canopy collapse or sudden wilt of plants. Presence of the disease has forced farmers to grow only 1 or 2 crops per year and to disinfest soils before each crop, rather than one application prior to the first crop. Normally, losses due to collapse of melon reach 5-10% of plants in the first crop and more than 50% in the second crop. The third crop results unfeasible. General disease symptoms are wilting subsequently followed by death of plants. The symptoms appear during harvesting or days before (8, 15). The syndrome has been described from melon growing areas worldwide, and may be caused by a complex of different pathogens (4, 24). Causal agents of vine decline diseases of melons include primarily *Monosporascus cannonballus* Pollack & Uecker (35), and *M. eutypoides* (Petrak) Arx (29), that are considered conspecific by Lovic *et al.* (22), *Macrophomina phaseolina* (Tassi) Goidanich (5), *Acremonium cucurbitacearum* A. Alfaro-Garcia, W. Gams et J. Garcia-Jimenez (13), *Rhizopycnis vagum* DF Farr (12, 26), *Melon necrotic spot virus* when associated with *Olpidium bornovanus* (Sahtiyanci) Karling (syn.: *O. radicale* Swartz & Cook fide Lange & Insunuza, *O. cucurbitacearum* Barr & Dias) (8, 34), and *Pythium ultimum* Trow and *P. aphanidermatum* (Edson) Fitzp. (16, 17). *Fusarium solani* (Mart.) Sacc. f. sp. *cucurbitae* (7). *Rhizoctonia solani* Künh also has been reported as a causal agent of sudden wilt of melons in Spain (6) and Israel (28). Diagnosis of the disease requires the detection and identification of the causal agent(s). *P. ultimum*, *P. aphanidermatum*, *A. cucurbitacearum*, *R. vagum* and *M. phaseolina* can be easily isolated from roots or crown on agar media (1, 14). *Monosporascus* is difficult to isolate from roots of symptomatic plants, but the perithecia visible on infected roots, are readily identified under a microscope (24, 25). To detect *O. bornovanus* it is necessary to examine the feeder roots under a light microscope, because as an obligate intracellular pathogen, it cannot be cultivated on synthetic media (14, 30). It is nearly impossible to obtain feeder roots from field diseased plants. Infection of plants with MNSV can be established using serological or PCR techniques on the stem tissue below the cotyledons of infected plants (8).

The objective of this study was to determine the presence of different fungi implicated in the collapse of melon in Honduras through the analysis of infested soils by 2 different techniques.

2. Materials and methods

2.1. Soil sampling and preparation of samples

Soil samples were collected from 30 soils, belonging to 6 different farms, plus two peat samples (one before and one after sown melon seeds that later showed collapse on the field), in the provinces of Choluteca and Ojo de Agua, Honduras. When symptoms of collapse were observed close to harvest of the first season crop, 10 soil samples (0-25 cm depth) from the root zone were randomly taken from each field, pooled, and thoroughly mixed. The resulting composite sample weighed approximately 1 kg. In the laboratory, each sample was put into a tray and covered with a filter paper to avoid air-borne contaminations and air-dried for 2 to 3 weeks. After screening through a 200 μm sieve, the fine fraction was used for dilution plating on acidified malt-extract agar (33). The coarse fraction was used to bait the fungi from soils using melon plants (9, 14).

2.2. Isolation directly from soils

A soil-dilution plate technique was used to isolate non-obligate pathogens that cause collapse of melons, such as *A. cucurbitacearum*, *M. phaseolina*, *R. vagum* and *Rhizoctonia solani* from soil (33). A 10 g subsample of the fine soil fraction (particle size less than 200 μm) of each sample was suspended in sterile water at 1 in 10^2, 1 in 10^3 and in 10^4 (w:w). After the suspensions were homogenized by hand-shaking for 4 min, 1 mL of suspension was transferred to a sterile Petri dish. Ten plates were used per dilution. Then, 10 mL per dish of acidified malt extract agar (Difco, U.S.A.) were added to each plate. The medium had been previously autoclaved (121ºC, 30 min) and left to cool to 32-35ºC. Petri dishes were then incubated in the laboratory. After 2, 5, 10 and 15 days the plates were examined for fungi. Plates containing less than 100 colony-forming units (CFU) were used for fungal population analysis. Morphological criteria were used to identify the different fungal colonies (2, 18, 19, 27, 36). Results are expressed as CFU per gram of soil.

2.3. Isolation with bait plants. The "soil phytopathometry" technique

We aimed to trap the fungi implicated in collapse of melon through analysis of roots of melon plants that had grown for 45 days in a 1:6 (w:w) mixture of soil sample and autoclaved (121ºC, 60 min) vermiculite. This bait-plant method was named "soil phytopathometry", and it was previously used to isolate *Fusarium oxysporum* f. sp. *melonis* from the xylem of wilting plants (3 and 4 weeks after sowing) (9).
The "soil phytopathometry" had 6 replicates per each of the 30 soil samples. Each replicate consisted of 25 g dry weight soil mixed with 150 g autoclaved vermiculite and added to a 1L plastic glass. Four 1 cm-diameter holes were made at 5 cm from the base of the glass. This was done to preserve moisture in the pot but avoid flooding. Seeds of the susceptible muskmelon cv. Amarillo-Canario (Ramiro Arnedo, Spain) were disinfested for 10 minutes in a 10% trisodium phosphate solution (Panreac Momplet and Esteban S.A., Spain), germinated in a moist chamber for 3-4 days, and then sown in the pots (6 seeds/pot). The glasses were randomly distributed in a climatic chamber (23 and 29ºC, 60-99% R.H., 16 hours 12 x 10^3 lux/day fluorescent radiation), and plants were hand-watered each 2 or 3 days with tap water and supplied with a complex fertilizer (Hakaphos Verde®, BASF, Germany). After 45 days (the time necessary to make sure that all the fungal pathogens had an opportunity to colonize the roots, and the MNSV was detectable on stems below cotyledons) the surviving melon plants were carefully removed from the pots and the roots washed free of soil and vermiculite. The roots were then analyzed depending on the pathogen (see below). Plants that died before 45 days were analyzed for non-obligated pathogens then. Two "soil phytopathometries" were done for those soil samples with enough amount of soil available after the first "soil phytopathometry". Negative controls consisted of pots filled with 175 g of sterile vermiculite. A positive control for *Acremonium* (a soil with a known history of *Acremonium* from Murcia, Spain) was included.

2.4. Analysis for Olpidium bornovanus

A 5 g sample from the roots of the 45-day-old plants were randomly collected from the roots of each replication. The samples were submerged in a 1.5% KOH (Panreac, Momplet and Esteban S.A., Spain) solution for 24 hours to clear the root surface, and then examined under light microscope to detect resting spores or sporangia of *O. bornovanus*. A total of 206 roots tangles corresponding with each "surviving" replicate were examined, 144 in the first "soil phytopathometry" and 62 in the second. Results were recorded as the number of positive roots tangles (replications) per total number of examined roots tangles.

2.5. Analysis for MNSV

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) serological analyzes were carried out with a specific MNSV polyclonal antisera (BIO-RAD, Life Sciences, Barcelona, Spain). From each glass, a mixture of all the crowns and roots was extracted with a 1:10 (p/v) buffer (PBST 1X pH 7,4 + 2% PVP). Positive and negative controls were provided by BIO-RAD. Two replicates were analyzed by ELISA. Tests were considered positive when the absorbance value was double the negative control. OD_{405} data where obtained with an ELISA Multiskan® EX Model nº 355 reader, using Ascent Software v2.6 (Termo Electron Corporation). Ambiguous results were repeated and analyzed by one-step reverse transcription-polymerase chain reaction (RT-PCR). ARN extractions were done from 0.1 g of roots. RNAwiz™ (Ambion Inc., Huntingdon, United Kingdom) was used. Tissue was ground with liquid N_2. SuperScript™ One Step IIRT/Platinum® Taq Mix (Invitrogen Life Technologies, Barcelona, Spain) was used. Samples were amplified with two specific MNSV coat protein primers (about 651 bp). A Mastercycler® personal 5332 eppendorf v1.3 (Eppendorf North America, Inc.) was used. RT-PCR products were purified with High Pure PCR Purification Kit (Roche Diagnostics, Manheim, Germany), and resultant sequences compared, using a BLAST Program, with GenBank MNSV Accesion No. DQ443545.

2.6. Analysis for Monosporascus cannonballus

The root system of each plant was examined under a dissecting scope (at 10x). When perithecia were observed, the species was identified by morphology and number of ascospores per asci as seen under higher (100x) magnification (19).

2.7. Analysis for non-obligated pathogens

For each plant (36 plants per sample and "soil phytopathometry" were analyzed), three rinsed and air-dried root pieces of 1 cm, showing browning, were placed on acidified malt-extract agar plates. The plates were examined after 2, 5, 10 and 15 days. This analysis allowed us to detect *Fusarium* spp., *Pythium* spp., *Acremonium* sp., *Rhizoctonia solani*, *Macrophomina phaseolina* and other fungi not associated with collapse of melons. The frequency of isolation of each fungus per sample was recorded. A total of 1,452 bait-plants were analyzed (139 of this plants belonging to controls).

3. Results

3.1. Isolation directly from soils

Analyzed soils yielded in general, a relatively wide variety of fungi despite the fact that the soils were disinfested annually. Fungi belonging to 17 genera were isolated. *Pythium* spp., *R. solani*, *M. cannonballus* or *R. vagum* were not detected from soils. Only *Macrophomina phaseolina* and *Acremonium* sp. were detected. *M. phaseolina* from 2 soils of different fields, and *Acremonium* sp. from one soil and from the used peat. *Fusarium roseum* (sensu Messiaen & Cassini), *Penicillium* spp., *Paecilomyces* sp., *Aspergillus* spp., *Curvularia* sp., *F. oxysporum* and *Rhizopus* sp., were most prevalent, occurring in more than 50% of the samples.

3.2. Isolation using the "soil phytopathometry"

Resting spores and sporangia of *Olpidium bornovanus* were observed in roots from bait melon plants grown in 21 of the 30 samples, representing all the studied fields. In 13 samples, this chytridiomycete was present in all of their replicates (Table 1). MNSV was detected from 23 samples, representing all the studied fields, and co-occuring with *O. bornovanus* in 22 samples. Controls were positive for MNSV in 19.4% and 25%, but never *O. bornovanus* (Table 1). Resultant PCR sequences confirmed the similarity with Gen Bank MNSV sequences. *Monosporascus cannonballus* was not observed on any root. Fungi of 14 different genera were isolated from roots of bait plants when plated on acidified malt-extract agar medium (Table 2). Neither *Acremonium cucurbitacearum* nor *R. vagum* were isolated from the 4,212 root pieces. From known fungi associated with vine decline of melon, *F. solani* was most frequently isolated, but the high frequency occurrence of this species in the controls complicates the interpretation of this result. Moreover, this fungus has not been reported as a vine decline pathogen on melon (7). *Pythium aphanidermatum* was the pathogen most frequently isolated by root plating, being isolated from 24 samples, representing all six studied farms and the peat before anf after use (Table 4). This oomycete was isolated mostly from plants (older than seedlings) that dead before 45 days. Low levels of *M. phaseolina* were found in 2 samples, from 2 different farms. *R. solani* was positive in 1 soil sample.

Table 1. Incidence of Olpidium bornovanus and Melon necrotic spot virus (MNSV) in roots of muskmelons grown in soil from melon production fields in Guatemala under controlled environmental environmental conditions.[a]

Sample	First "Soil Phytopathometry"		Second "Soil Phytopathometry"	
	O. bornovanus	MNSV	*O. bornovanus*	MNSV
1[c, I]	2/3[b]	2/3	3/5	1/5
2[I]	4/5	3/5	6/6	6/6
3[I]	5/6	3/4	-	-
4[I]	6/6	4/6	4/5	4/6
5[I]	5/6	5/5	-	-
6[I]	1/2	4/4	-	-
7[I]	5/5	2/5	-	-
8[I]	1/1	-	-	-
9[II]	6/6	0/6	5/6	0/6
10[II]	2/2	2/2	-	-
11[II]	0/6	1/6	1/6	1/6
12[II]	1/1	0/2	1/1	-
13[III]	4/4	3/4	-	-
14[III]	6/6	3/6	-	-
15[III]	6/6	1/6	-	2/3
16[III]	6/6	0/6	-	6/6
17[III]	5/5	-	-	4/5
18[III]	0/5	1/5	6/6	2/6
19[Peat before use]	3/6	0/6	0/6	3/5
20[Peat after use]	5/6	3/6	1/6	0/6
21[IV]	3/3	2/3	-	2/3
22[V]	-	2/5	-	-
23[V]	-	1/1	-	-
24[V]	-	2/2	-	-
25[VI]	-	3/4	-	-
26[VI]	0/1	0/1	-	-
27[VI]	-	1/1	-	-
28[VI]	0/4	0/4	-	-
29[VI]	0/2	0/2	-	-
30[V]	0/5	1/5	-	-
Control	0/36	7/36	0/12	3/12

[a] Soil from each sample was diluted 1:6 with sterile vermiculite and added to a 1L plastic cup, where six seeds of the susceptible muskmelon cv. Amarillo-Canario (Ramiro Arnedo, Spain) were sown. Six replicates (glasses) per sample were randomly distributed in a climatic chamber (23 and 29ºC, 16 hours 12 x 103 lux/day fluorescent radiation) for 45 days. Roots of these plants were analyzed for O. bornovanus and a mixture of crown and roots for MNSV.

[b] Replications with the pathogen/total number of replications

[c] Samples with the same superscript (I, II, III, IV, V, VI) were taken from the same farm.

Table 2. Fungi isolated from roots of muskmelons grown in soil from melon production fields in Honduras under controlled environmental conditions.[a]

	Samples from which fungus was baited [b]	Farms and peats from wich fungus was baited [c]	Frequency of isolation [d]	Frequency of isolation from controls (%)
Paecilomyces	27	8	15.9	38.12
F. solani	26	8	35.2	7.91
Pythium	24	7	45.8	0
F. roseum [v]	20	8	6.3	5.04
Penicillium	16	7	12.1	2.16
F.moniliforme	16	8	5.93	29.50
Curvularia	11	6	1.33	0
Trichoderma	10	5	4.53	0
Cladosporium	10	8	2.27	0
F. oxysporum	9	5	2.93	2.88
Aspergillus	9	5	1.7	2.88
Rhizopus	5	5	0.37	2.88
M. phaseolina	2	2	0.67	0
Gliocladium	1	1	0.39	0
R. solani	1	1	0.13	0
F.dimerum	1	1	0.1	0
Alternaria	1	1	0.1	0
S. rolfsii	1	1	0.07	0
Mycelia sterile	1	1	0.07	0

[a] Soil from each sample was diluted 1:6 with sterile vermiculite and added to a 1L plastic cup. where six seeds of the susceptible muskmelon cv. Amarillo-Canario (Ramiro Arnedo. Spain) were sown. Six replicates (glasses) per sample were randomly distributed in a climatic chamber (23 and 29ºC. 16 hours 12 x 103 lux/day fluorescent radiation) for 45 days. Three pieces of roots of each plant were plated on malt extract agar.
[b] From a total of 30 soil samples.
[c] From a total of 6 fields and two peats.
[d] Values are the average of the frequency of isolation of 30 samples. expressed as proportion (%) of positive plants per sample.
[e] F. roseum was represented by *F. equiseti* (>90% isolates). *F. semitectum*. *F. chlamydosporum* and *F. acuminatum*.

4. Discussion

We sought to determine the presence of different causal agents of vine decline diseases in 30 soils from 6 different fields of Honduras where collapsed plants were observed, by using two different techniques. The first technique consisted on direct isolation of fungi by plating suspensions of soil in a general agarized medium, and the other one was a bait plant method named "soil phytopathometry", previously used to isolate *F. oxysporum* f. sp. *melonis* from soils of Mexico (9).

Results of our investigation prove the consistent association of *Olpidium bornovanus* and MNSV with vine decline in Honduras fields. All studied farms, and 70% of all soil samples, tested positive for *O. bornovanus*. MNSV was also present in all studied farms, and 76.7% of samples, co-occurring with the chytridiomycete at 95.2% of *O. bornovanus*-positive samples.

Detection of other fungi implicated in the collapse syndrome, in the same soils, was lower. *Pyhium aphanidermatum* was found in 24 samples, representing all farms. *M. phaseolina* was found in 2 different farms.

R. solani was isolated from only 1 field. *M. cannonballus*, *R. vagum* and *Acremonium* were never isolated from the soil samples using bait plants or the soil-dilution plate technique.

O. bornovanus and MNSV were previously reported from Guatemala by Jordá et al. (20). This co-occurrence of fungus and virus was considered to be the main reason for the collapse of melons in Almería, Southern Spain (8) and Sardegna, Italy (34). Bruton and Miller (4) analyzed collapsed muskmelons from 12 fields of Honduras and found *R. solani* the most frequently isolated fungus form roots and crown. They also isolated *F. semitectum* and *F. solani* from more than 30% of analysed plants; *Pythium* sp. from more than 50% of plants from one farm, and *M. phaseolina* from 65% from another farm; they isolated *R. vagum* from one farm; and *M. cannonballus* was infrequently isolated from only one parcel of one farm.

Our work shows a technique using bait plants ("soil phytopathometry") that allowed us to diagnose several organisms involved in vine decline diseases. The diagnosis of vine decline of melon is not easy, due to the diversity of possible pathogens involved, and that root systems of collapsed melons generally lack tertiary and feeder roots, where *Olpidium* must be observed (30), and remaining roots are often rotting, supplying a medium for saprophytic fungi that can hide the presence of causal agents of collapse (21). Sometimes, a few distinctive signs, such as perithecia of *M. cannonballus* on secondary and tertiary roots, or microesclerotia of *M. phaseolina* on the crown, may aid in the diagnosis, but in the absence of such signs, observation of fungal sporulation on surface-sterilized tissue or isolation of the fungus in culture, is needed (1). Ascospores of *M. cannonballus* can be recovered by a quantitative method (32) and *M. phaseolina* can easily be isolated from soils with soybean and maize stems (10). Carnation petals and other plant material are good traps for *Pythium aphanidermatum* occurring in soil or water (33). However a general method allowing the detection from soils of all these pathogens, is lacking.

"Soil phytopathometry" technique provided us a 45-day-old melon plants with the root system full of tertiary and feeder roots, and enough time to plants trap the fungi and the virus. With the exception of *R. vagum*, in the "soil phytopathometries" there was, at least, one sample showing any of the CAC. *Acremonium* was isolated from its positive control, and perithecia of *M. cannonballus* were observed on 45-days-old roots belonging to 3 different samples. One of these samples was analyzed by "soil phytopathometry" using diverse soil dilutions, and *M. cannonballus*' perithecia were observed on 45-days-old roots at 1:7.5 and 1:10 (w:w) soil:vermiculite proportion, being not observed at 1:15 nor 1:30 (data not shown). This result indicates that dilution may affect diagnosis for *M. cannonballus*, as an outcome of inoculum density in the analyzed soil. *M. cannonballus* in naturally infested soils has been reported to infect the roots from between 9 to 17 days after planting in summer-autumn season, and from 35 to 36 days in late winter-spring (31). Waugh et al. (37) reported from in vitro studies that perithecia of *M. cannonballus* were first produced between 14 and 20 days after planting, with up to 45 perithecia per cm^2 after 27 days at 25ºC. After seven weeks, mature perithecia were observed on roots when melon plants were inoculated with different isolates of *M. cannonballus* (21). It is also known that *O. bornovanus* colonizes the roots of melons in less than 2 weeks, even when an infective soil was 1:100 diluted in sterile sand (11, 15). *M. phaseolina* was easily isolated from beet root seeds only 4 days after inoculation, and 15 days from soybean and maize stems (10). Garcia-Jimenez et al. isolated *Acremonium* from roots of muskmelon forty days after inoculation (13). For reliable detection of MNSV infection from stem tissue below the cotyledons of susceptible melons, a forty day growth period is sufficient (23). An additional observation is the fact that melon seeds can carry the MNSV. In our study, controls were positive in a similar proportion to those previously reported (11, 15). When MNSV was present in bait plants from soil samples, the proportion of positives was higher than controls, so we can consider that seeds weren´t the primary inoculum source for these soils.

The another technique used, soil-dilution plating method, allows isolation of fungi with saprophytic activity (33). Despite regular soil disinfestation, many species of fungi were present. Some species belonged to Ascomycetae (*Phoma*, *Penicillium*, *Paecilomyces*, *Aspergillus*, …), other to Zygomycetae (*Rhizopus*) and to Deuteromycetae. These fungi may inhibit the growth of CAC on the plates. Dhingra et al. reported *M. phaseolina* to be a poorly competitive saprophyte (10), but it was the only fungus associated with vine decline of melons that could be isolated directly from soils. *Pythium aphanidermatum*, like the other fungi except *M. phaseolina*, was never isolated using this soil-dilution technique, but it was frequently isolated using the plant bait technique. Tello and Lacasa (33) reported similar results from Spanish soils, and assumed that the soil-dilution technique favours fungal spores, not mycelium fragments, to form colonies on the agar medium. It can also be hypothesized that the time between the wetting of dried soil and plating onto the medium may be insufficient to re-hydrate and activate *Pythium* spores. Definitively, the soil-dilution technique is not a good method to isolate causal agents of collapse from soils, despite of their known saprophytic activity.

In conclusion, "soil phytopathometry" seems a good technique to detect the presence of different causal agents of vine decline of melon, specially the obligate pathogen *O. bornovanus* and MNSV. By this technique, these two organisms were consistently associated with the presence of collapsed melon plants in Honduras. To our knowledge this is the first report of such association in this Country.

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Effect of nitroaromatic compounds on the growth of potted plants

L. Dubova[1,2], **B. Limane**[2], **O. Muter**[2,*], **A. Versilovskis**[2], **Dz. Zarina**[2], and **I. Alsina**[1]

[1] Latvia University of Agriculture, 2 Liela Str, Jelgava LV-3001, Latvia
[2] Institute of Microbiology and Biotechnology, University of Latvia, 4 Kronvalda blvd, Riga LV-1586, Latvia

[*]Corresponding author: e-mail: olga.muter@inbox.lv, Phone: +371 67033897

Effect of nitroaromatic compounds to higher plants was studied using wheat, barley, tomato, radish, cress salad as test-organisms. Additionally, *Koeleria glauca,* which is naturally disseminated at the polygon in contaminated soils, was sampled and used in vegetation experiments. Soil and the mixture of nitroaromatics (brown powder remaining from the partial detonation of munition) were sampled at the military polygon. A regular addition of an equivalent dosage of nitroaromatics (i.e. total amount 8.54 mg nitroaromatics/kg soil) for potted plants during two-month vegetation experiment was provided. Two-month treatment of wheat, barley and radish with BP resulted in an enhanced growth, i.e. their shoot height was 62 %; 67 %; and 36 % higher, correspondingly, as compared to control samples. In turn, tomato and cress salad seedlings were inhibited by BP up to 62 % and 80 %, correspondingly. Among tested plants, cress salad demonstrated the highest sensitivity to nitroaromatics in both, 4-day root elongation test and 58-day vegetation experiment.

Keywords nitroaromatic compounds; potted plants; toxicity; root elongation test

Abbreviations BP - brown powder; NA – nitroaromatic compounds

1. Introduction

Pollution of soil by explosives is a serious environmental problem. Site investigations indicate that TNT is the least mobile of the explosives and most frequently occurring soil contamination problem. Toxicity of NA, i.e. 2,4,6-Trinitrotoluene and its primary reduced metabolites, for biota has been shown in experiments with different organisms [1-3]. However, a wide spectrum of NA, a diversity of test-organisms, as well as physico-chemical factors, which synergistically affect organism in the presence of NA, make toxicity evaluation rather difficult.

Seed germination and plant growth bioassays are the most common techniques to evaluate soil phytotoxicity [4]. Plant growth is considered to be a more sensitive parameter than the seed germination. Probably, the great quantity of nutrient reserves in seeds of some plant species would have lower sensitivity to toxicity [5,6].
Under natural conditions, NA become bioavalilable only in limited extent, due to their low solubility in water. The fates of different explosives in plants are various. RDX (1,3,5-trinitroperhydro-1,3,5-triazine) has limited metabolism in plants, TNT was rapidly reduced in to hydroxylamines and conjugated to sugars by plants [7].

The aim of this study was to compare different plant species (both, monocotyledons and dicotyledons) in their response to soils contaminated by nitroaromatic compounds. Statistically significant difference in this response could provide valuable information on interrelations between higher plants and explosives.

2. Materials and methods

Non-contaminated coarse sandy soil for vegetation experiment was collected at the military polygon. Brown powder was sampled at the same polygon and later identified by HPLC as a mixture of NA. Further measurement of the concentration of explosives in soil samples was performed according to EPA 8330 [8-9].

Vegetation experiment was established in July-August 2006 and performed with: wheat (*Triticum aestivum*); barley (*Hordeum vulgare*); tomato (*Lycopersicon esculentum* L.), radish (*Raphamus sativus*), cress salad (*Lepidium sativum*). Plastic pots with capacity of 800 ml were used, each receiving 10 seeds. Each plant species were sown in two pots. One of them was treated with water, brown powder solution and fertilizer, another only with water and fertilizer (control). Vegetation experiment was carried out in field conditions during 58 days. Temperature ranged between 21 to 26 °C at day time and 15 to 18 °C at night time during experiment. No precipitation. Kemira Horti (Kemira GrowHow, Finland) fertilizer was used for plant fertilisation with the following content, mg/l: N – 80; P – 80; K – 60; B – 0.1; Cu – 0.1; Fe – 0.2; Mn – 0.1; Mb – 0.05; Zn – 0.05. A volume of unit-dose of the fertilizer was 30 ml. Treatment by brown powder was performed every second day, using stock of BP. The total amount of NA received by one pot during experiment was 8.54 mg/kg soil.

Biotests on seed germination were performed according EPA 712-C-96-152 guidelines, in triplicate, with the same plant species used in vegetation experiment.

For determination of the total bacteria count Tryptone Glucose Yeast Extract Agar (Sifin, Germany) was used. The M8 medium amended with 50mgBP/l was used as a selective medium for explosives-degrading bacteria [12]. Samples were plated in triplicate. Cultivation was performed at +28 °C, for 48h (for TGA) and 96h (for M8 amended with BP).

3. Results

A brown powder was sampled at the military polygon and further identified by HPLC as a mixture of nitroaromatic compounds and used for plant toxicity studies (Table 1). Testing of BP showed that the 4-Amino-2,6-Dinitrotoluene was in a higher concentration as compared to other compounds, which is known as the most commonly identified transformation product during 2,4,6-Trinitrotoluene biodegradation [3]. Current content of BP can be a result of incomplete detonation and partial biodegradation.

Table 1 Composition of brown powder identified by HPLC.

Peak name	Amount, µg/ml
1,3-Dinitrobenzene	17.384
1,4-Dinitrobenzene	3.974
2-Amino-6-Nitrotoluene	0.129
4-Amino-2-Nitrotoluene	2.719
4-Amino-2,6-Dinitrotoluene	8 672.557
2,4-Dinitrotoluene	29.965
2,6-Dinitrotoluene	9.614
3,4-Dinitrotoluene	22.636
2-Nitrotoluene	1.237

3.1 Effect of brown powder to the root elongation of various plant species

Preliminary experiments were performed with the aim to reveal the effect of brown powder to various plant species by root elongation test. Brown powder in the concentration range 0.4 ÷ 13 mg/l affected the growth of seedlings in different manner. Thus, tare, barley, wheat and peas were shown to be the most resistant to the tested range of BP concentrations. Moreover, at the lowest BP concentration tested in this experiment, tare was stimulated by BP, as compared to the control sample without NA. In turn, radish, cress salad, flax, lupin and red clover were inhibited in concentration dependent manner (Fig.1).

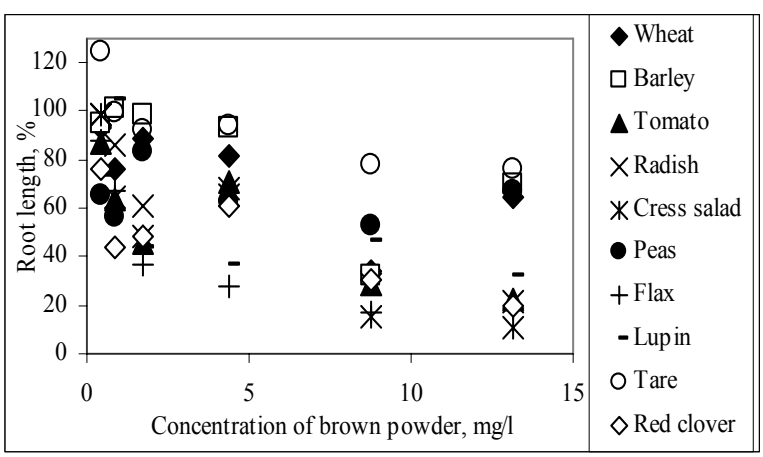

Fig. 1. Effect of brown powder on the root elongation of various plant species. Data on the changes of root length in dependence on concentration of BP is expressed in %, thus as 100% was calculated the root length of a seedling incubated in water without BP.

3.2 Effect of brown powder to the various plants during long term vegetation experiment

During two-month vegetation experiment with potted plants the effect of BP to tested plants was different. The measurements of plant weight, dry weight and height were performed (Fig.2a, 2b, 2c).

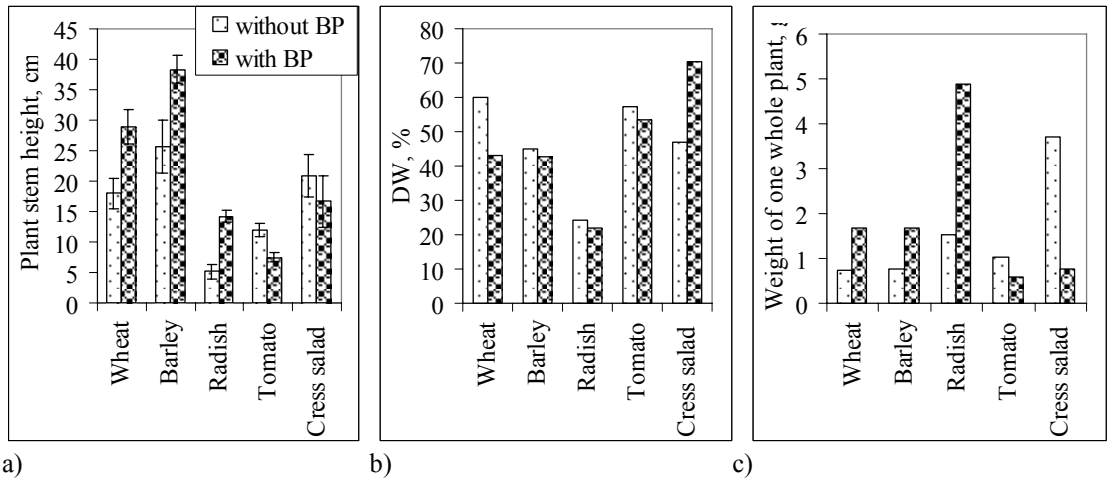

Fig. 2 Effect of brown powder to the growth of tested plants during 58 days vegetation experiment. Measurement of stem height (a); dry weight of the plant (b); wet weight of the plant (c).

As shown in Figure 2a, treatment of wheat, barley and radish with BP resulted in an enhanced growth, i.e. their shoot height was 62 %; 67 %; and 36 % higher, correspondingly, as compared to control samples. In turn, tomato and cress salad seedlings were inhibited by BP up to 62 % and 80 %, correspondingly (Fig.2a). The results on dry and wet weight of tested plants showed their dependence on BP treatment. (Fig.2b and 2c). Dry weight of green biomass for all tested plants was slightly decreased in the samples treated with BP, except cress salad. These results indicated to the differences in plant tissue structure caused by BP treatment. According to Kapustka, the total dry matter provides the best indication of an adverse plant response to toxic substances [5, 11].

Among plant species tested in the long-term vegetation experiment and root elongation test (3.1.), four plants, i.e. wheat, barley, tomatoes and cress salad, demonstrated the similar tendency in terms of the resistance to nitroaromatics. Thus, a response to the presence of nitroaromatics is species-specific, and a search for appropriate test-organism for toxicity testing needs a preliminary screening of various plants [4].

3.3 Changes of microbial count in soil samples after vegetation experiment with brown powder treatment

Regarding the changes of microbial community in soil samples after 58 days vegetation, there are some considerable changes occurred in dependence on plant species (Fig.3). For example, soil treatment by BP during growth of wheat resulted in increased microbial count, i.e. the total count in non-treated and BP-treated soils with wheat was 3.8×10^5 and 9.6×10^6 cfu/g soil, correspondingly. Besides, the growth of tomato resulted in the total inhibition of bacteria grown in selective medium amended by BP. It attributes to the both, non-treated and BP-treated samples. Other tested plants did not show an inhibition effect to the bacteria, which can use NA as a sole nitrogen source.

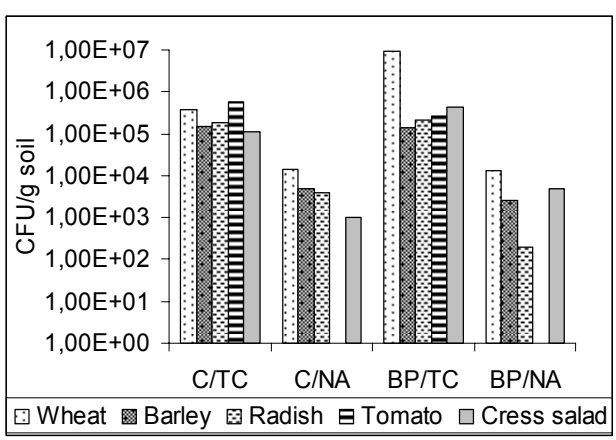

Fig. 3 Changes of microbial count in soils after vegetation experiment with various plant species. C/TC – the total bacteria count of the control soil sample, i.e. without treatment with brown powder (BP); C/NA – the number of bacteria grown on selective medium, i.e. with BP as a sole nitrogen source (control sample); BP/TC - the total bacteria count in the soil sample treated with BP; BP/NA - the number of bacteria grown on selective medium, i.e. with BP as a sole nitrogen source (sample treated with BP).

3.4 Growth of plants in the freshly contaminated soils under natural conditions

Visual inspection of flora distribution near detonation crater at the military polygon provided additional information on plants resistance to toxic explosives. For example, *Koeleria glauca* was the sole plant species, which grew close to detonation crater in the coarse sandy soils contaminated by explosives. This fact could indicate to the resistance of this plant to explosives and its further use in phytoremediation process. However, testing of soils before and after treatment by BP showed an increased concentration of NA after treatment by BP in the samples with *K. glauca* (results not shown). It could indicate to the resistance mechanism to NA for *K. glauca*, which prevents an uptake of these compounds by a plant. If this hypothesis will be proved in further experiments, it could be concluded about unefficiency of *K.glauca* in phytoremediation process for soils contaminated by nitroaromatics. On the other hand, this plant could be a valuable model for a study on the mechanisms of plant resistance to nitroaromatic compounds.

4. Discussion

Summarizing the results obtained in this study it could be concluded, that among tested plants, cress salad remains to be one of the most sensitive plant to NA and, therefore, appropriate test-organism for assessment of soil phytotoxicity.

Stimulating effect of NA for wheat, barley and radish needs to be studied in future experiments to reveal the processes occurred during long term interrelation between NA and plant. As Gong et al. reported, seed germination and early stage seedling growth tests showed the TNT stimulating effect for cress salad and turnip in the concentration range 5-25 mgTNT/kg soil, and for oat and wheat in the concentration range 25-50 mgTNT/kg soil [12]. The same range of NA concentrations was studied in our experiments. However, cress salad did not exhibit an enhanced growth in the presence of nitroaromatics. Another study on TNT toxicity for higher plants was reported by Robidoux et al. After 14-day experiment, growth of barley was found to be considerably reduced in the presence of 56 mgTNT/kg artificially spiked silica [13]. Most probably, there is a difference between an effect of pure TNT and mixture of nitroaromatics to plant growth. Besides, toxicity of any tested compound can differ in dependence on the plant development stage. An experimental design also plays a crucial role in toxicity assessment [14]. New series of experiments with higher NA concentrations could reveal the toxic level of NA for those plant species, which demonstrated the resistance in the present study.

A special attention in future experiments should be paid to the changes in soil microbial community caused by acute or chronic contamination, remediation activities, and/or plant species grown in tested soil. Antagonistic interrelations between tomato plants and nitroaromatics-degrading bacteria indicated to the importance of interrelations between soil, contaminant, plant and microorganisms in terms of toxicity and remediation. Further experiments with *Koeleria glauca* could provide additional data on resistance mechanism of this plant, which plays a “pioneer” role in the soils have been freshly contaminated by explosives.

Acknowledgements Work was supported by contract AĪVA 2004/288 from the Ministry of Defense, the Republic of Latvia. Work was partially supported by the Projects 04.933 and 04.1046, Latvian Council of the Science. We are grateful to Anna Zheiviniece for consulting in plant identification. We thank National Armed Forces of the Republic of Latvia for providing chemicals, as well as consulting and assistance in soil sampling at the polygon. We also acknowledge the helpful discussions of Dr.Chem.Vadim Bartkevich from the National Diagnostic Centre.

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Effects of cattle grazing, trampling and excrement deposition on microbial nitrogen transformations in upland soil

M. Šimek*, J. Čuhel

Biology Centre of the Academy of Sciences of the Czech Republic, v.v.i. - Institute of Soil Biology, and University of South Bohemia, Faculty of Science, Na Sádkách 7, CZ 370 05 České Budějovice, Czech Republic

*Corresponding author: e-mail: misim@upb.cas.cz, Phone: +420 385310174

Rates of selected soil nitrogen transforming processes as well as kinetic parameters of denitrification enzymes were determined in a cattle overwintering area. Soils from three localities differently impacted by the cattle (severe, moderate, control with no impact) were examined. In cattle-influenced soils, total N, organic C and pH were significantly increased. Consequently rates of potential mineralization, nitrifying enzyme activity and denitrifying enzyme activity (DEA) were enhanced compared to control, while potential nitrogenase activity was lowered in both soils influenced by cattle. The soils differed substantially in DEA, which was about 60 ng N_2O-N g dw^{-1} h^{-1} in control soil but 3 times and 34 times higher in moderately and severely impacted soils, respectively. The soils also exhibited significantly different maximum reaction velocity (V) and Michaelis-Menten constant (K_m) of enzymes responsible for reduction either of nitrate to nitrous oxide or of nitrous oxide to di-nitrogen. Results suggest that the cattle-induced stress alters the functioning of soil microbial community.

Keywords nitrogen transformations; denitrification; enzyme activity; soil; pasture; cattle

1. Introduction

Soil microorganisms in general and those involved in nitrogen transformations in particular are of a great importance for grassland ecosystem functioning as they determine nutrient availability in soil as well as release and losses of nitrogen from the system. In contrary, grassland management affects activities and composition of soil microbial communities due to a number of mechanisms related to mowing and grazing events. In grazed grasslands, animals not only substantially influence vegetation cover, but could also have various effects on soil organisms and biological processes.

In cattle husbandry in the Czech Republic, typical pasture management in marginal regions with low-input agriculture includes overwintering of the animals. For this purpose, a specific pasture site, called overwintering area, is used. It is typically located near the barn and used during the winter season by the cattle herd; the animals have free access either to the barn or to the overwintering area. The overwintering period can last from the end of October to the middle of May. Soils in cattle overwintering areas are unique in that they are exposed to unusually high animal physical impacts (trampling/compaction) and elevated N inputs in the form of dung and urine, resulting in soil structure damage and N-saturation conditions, respectively. Moreover, most N is deposited in the overwintering area during the winter season, when plant nutrient uptake is greatly lowered and soil processes are also less active. As a consequence, cattle overwintering areas have been identified as important hot spots of gaseous emissions including emissions of nitrous oxide [1, 2].

At our experimental area, gradient of animal impact exists as a result of ca 10-y management practice. Some localities of the area, e.g. near the barn, have been enriched by great amounts of nutrients in deposited excrements and also soil structural properties were substantially modified. Accordingly, we proposed the following hypothesis: due to nutrient inputs highly exceeding that in an ordinary pasture, and due to specific spatial distribution of localities differing in the animal impact cattle, overwintering areas represent an appropriate model site for studies on cattle impact on soil microbial community. In this paper we report data obtained during several-year period in a cattle overwintering area located in South Bohemia, Czech Republic. Our objective was to determine rates of important nitrogen transformations in soils differing in the extent of cattle influence and to evaluate the impact of cattle on soil microbial consortium.

2. Material and methods

2.1 Study area and soils

Soils were sampled at Borová Farm near Český Krumlov in South Bohemia, the Czech Republic (altitude 630 m, latitude 48°52′ N, longitude 14°13′ E). Since 1995, the approximately 4 ha pasture had been used for

overwintering of about 90 cows. Three localities were identified within the area differing in the rate of animal impact: severely impacted locality characterized by totally destroyed plant cover and completely disturbed topsoil (No 1 here), moderately impacted locality (No 3), and control undisturbed locality (No 5).

The soil at the area was originally classified as Cambisol (sandy loam with 80% sand, 14% silt and 6% clay) but recently identified as Haplic Phaeozem (arenic) by the WRB system (M. Yli-Halla, personal communication). Topsoils (0-15 cm) were collected in October 2004 and used for the determination of kinetic parameters of denitrification enzymes. Other soil samples were taken from 4 layers (0-5, 5-10, 10-15, and 15-20 cm) in May 2005 and used for most analyses described. The three subsamples for each locality/soil layer were mixed together, sieved (4 mm) and stored at field moisture in plastic bags at 4°C on the day of sampling until used. Soil properties are provided in Table 1.

Table 1 Basic properties of the soils under study sampled from 0-5 cm layer. Except the pH values, the data apply for soils sampled in 2002 [1]. Means from 12 or 4 (C_{org}, N_{tot}) values. Different letters behind the means indicate significant differences among the soils.

soil	C_{org} (% w/w)	N_{tot} (% w/w)	C_{mic} (μg C g dw^{-1})	bulk density (g cm^{-3})	porosity (%)	WFPS (%)	pH (H_2O)
No 1	5.17 a	0.50 a	1415 a	1.08 a	54.7 a	74.6 a	8.4 a
No 3	3.35 b	0.30 b	495 b	1.04 a	58.1 a	65.6 b	7.4 b
No 5	2.19 b	0.27 b	440 b	1.32 b	45.6 b	81.9 a	6.3 c

Note: Organic carbon (C_{org}) was determined by wet oxidation with acid dichromate, total nitrogen (N_{tot}) by Kjeldahl digestion, microbial biomass C (C_{mic}) was estimated with $CHCl_3$ fumigation extraction [3] followed by dichromate digestion of extractable C. Bulk density, porosity and water-filled pore space (WFPS) were determined as described by [4]. Soil pH was measured in a 1:2.5 (w:w) soil to water mixture.

2.2 Determination of rates of N-transformations

Rate of potential nitrogen mineralization was determined in soil subsamples incubated at 25°C in dark in anoxic conditions for 7 days [5]. Denitrifying enzyme activity (DEA) was measured using short-term assay by [6] as described by [1]. Nitrifying enzyme activity (NEA) was determined in soil subsamples incubated at 25°C in oxic conditions for 8 hours [7]. Potential nitrogenase activity was measured as acetylene-ethylene reduction activity [8] during 24-h incubation at 25°C in dark in oxic conditions.

2.3 Determination of kinetic parameters of denitrification enzymes

Before the experiments all NO_3^- was removed from the soils due to facilitating a direct determination of kinetic constants of N_2O reductase as follows: soil slurries (10 g fresh soil, 25 ml H_2O) in 330 ml bottles were supplied with 1 g anion exchange resin A-520E (Purolite International, UK) in permeable pocket, which was removed from the slurry after 1 h shaking (150 rev min^{-1}, 25 °C). The anion exchange resin removed 94.3 – 94.8 % NO_3^-. Further, the bottles were capped with rubber stoppers and metal holders, made anaerobic (4 cycles of evacuation and flushing with 0.15 MPa He, lasting in total 16 min), 1 ml deoxygenated solution of glucose (6875.0 mg l^{-1}) was added and the bottles were then incubated 10 h while shaking at 25°C. The time period of 10 h was determined in preliminary experiments, where the bottles were provided with 30 ml C_2H_2 and N_2O concentration in headspaces was measured (using GC) until stopped to increase (data not shown).

After the NO_3^- removal and making the bottles anaerobic again (as described above) the kinetic parameters of denitrification enzymes were determined. Maximum reaction velocity (V) of NO_3^- reduction to N_2O was determined according to [9]; however KNO_3^- solution had to result in higher final NO_3^- concentration in the slurry (3 mM) compared to the concentration cited in the reference (1 mM), as revealed in preliminary experiments.

Maximum reaction velocity (V) and Michaelis-Menten constant (K_m) of N_2O reduction to N_2 were determined according to the modified method of [9]. Sets of 24-27 bottles (for each soil) were loaded with different N_2O doses (0.2 – 4.3 μM) and shaked at 25°C. Four gas samples were periodically taken from each bottle during a 30-min (for the soils 3 and 5) or 6(9)-min (for the soil 1) incubation and N_2O concentration in the gas samples was quantified with GC. The N_2O reduction rates for each initial concentration and V and K_m values for N_2O reductase were calculated according to [9].

3. Results and discussion

The soils differed substantially in many characteristics; the soil 1 exhibited much higher organic matter content and microbial biomass carbon than the two other soils, which corresponds to severe animal impact (Table 1). It is further documented by significantly increased pH both in the soils 1 and 3, typically related to the presence of animals (due to excretal returns). Surprisingly, the highest bulk density and the lowest porosity were indicated for the soil 5, which could be due to much lower content of organic matter in this soil; soil organic matter is crucial for soil structure formation and maintenance [10].

Average rates of nitrogen transformation processes determined at laboratory-incubated soil samples are shown in Table 2. Nitrogen mineralization rates were the highest in surface 5 cm layers in all the soils and decreased in soil profile. Both soil 1 and 3 had significantly higher nitrogen mineralization in comparison with control soil 5. The same tendencies were seen also in nitrification and denitrification activities: they were decreasing in deeper layers in all soils, and cattle impacted soils displayed much higher activities than control soil. The only exception was denitrifying enzyme activity in soil 1, which was even higher in the layer 5-10 cm than in the surface 0-5 cm layer.

In contrary to other three enzymatic activities, nitrogenase activity was the lowest in surface layer and increased in deeper layers of the soils 3 and 5. It was also much higher in the control soil 5 and less impacted soil 3 in comparison with the soil 1. Specific distribution of nitrogenase activity in soil profiles as well as the difference between control soil and severely impacted soil could be however expected - nitrogenase activity should be decreased in the soils rich in mineral nitrogen (that is in the soil 1 and partly 3 which are enriched with nutrients from excrements), as di-nitrogen fixing microorganisms are in general suppressed by increased concentrations of soil nitrogen.

Estimated kinetic parameters from the different experiments are summarized in Table 3. The soils exhibited quite different V for reduction of NO_3^- to N_2O; the value for the soil 3 was approximately 10-times lower than for the soil 1, and the soil 5 exhibited even lower value (0.4 μg N g dw^{-1} h^{-1}) than the soil 3. The results for V of reduction of N_2O to N_2 were similar; the values for the soil 1 were clearly much higher than for the soils 3 and 5, and were the lowest for the soil 5. The K_m values of reduction N_2O to N_2 showed small to none differences between the soils 3 and 5 (1.0 and 0.9 μM N, respectively) and were lower than in the soil 1 (1.3 μM N).

To asses physiological differences between the soils, the ratios between V for the two processes (V NO_3^- reduction/V N_2O reduction, [9]) were examined due to better understanding the role of the denitrifying consortia in regulating the N_2O emissions (the V for both reactions can be affected by the amount of denitrifying microorganisms and so by the amount of reductases). The soil 1 exhibited the highest ratio among the three soils while the ratio for the soil 5 was the lowest.

Table 2 Rates of nitrogen transformations in the soils under study. Means from 4 (NEA, DEA) or 3 replicates, standard deviations in parentheses.

Soil	Soil layer (cm)	Nitrogen mineralization (μg N g dw^{-1} d^{-1})	Nitrification (NEA) (μg N g dw^{-1} h^{-1})	Denitrification (DEA) (ng N g dw^{-1} h^{-1})	Nitrogenase activity (nmol ET g dw^{-1} h^{-1})
No 1	0 – 5	6.227 (0.599)	1.290 (0.082)	5813.3 (239.2)	11.53 (1.59)
	5 – 10	4.837 (0.094)	0.950 (0.065)	7227.9 (1708.1)	12.13 (0.31)
	10 – 15	2.523 (0.031)	0.866 (0.217)	3836.9 (419.3)	12.49 (3.46)
	15 - 20	1.876 (0.175)	0.981 (0.040)	3058.2 (226.9)	4.47 (1.37)
No 3	0 – 5	5.191 (0.104)	0.943 (0.194)	3266.7 (514.0)	11.21 (0.56)
	5 – 10	1.728 (0.197)	0.433 (0.104)	932.3 (127.1)	11.97 (0.56)
	10 – 15	0.870 (0.082)	0.313 (0.105)	298.6 (111.3)	18.22 (3.09)
	15 - 20	0.531 (0.116)	0.203 (0.172)	85.6 (26.1)	38.71 (3.05)
No 5	0 – 5	3.559 (0.492)	0.395 (0.186)	1873.0 (440.9)	21.11 (1.87)
	5 – 10	2.077 (0.079)	0.125 (0.018)	452.0 (110.5)	27.01 (1.08)
	10 – 15	0.772 (0.154)	0.045 (0.011)	145.8 (19.3)	46.25 (5.22)
	15 - 20	0.511 (0.118)	0.032 (0.018)	53.3 (13.9)	97.48 (6.49)

Table 3 Estimated kinetic parameters V and K_m of NO_3^- and N_2O reductions for the soils 1, 3 and 5 (standard error of means in parentheses).

Soil	NO_3^- reduction	N_2O reduction		Calculated ratio
	V	V	K_m	V NO_3^- red./V N_2O red.
	μg N g dw^{-1} h^{-1}	μg N g dw^{-1} h^{-1}	μM N	
No 1	12.0 (0.3)	16.6 (1.5)	1.3 (0.3)	0.7
No 3	1.1 (0.1)	2.7 (0.3)	1.0 (0.3)	0.4
No 5	0.4 (0.0)	1.6 (0.2)	0.9 (0.2)	0.3

4. Conclusion

Results show clear cattle-induced effects on key soil processes of microbial nitrogen transformations in the overwintering area and suggest that the cattle-induced stress alters the functioning of soil microbial community in the upland soil.

Acknowledgements The research was supported by the GA AS CR (No IAA600660605), the Research Plan of the Institute of Soil Biology (No AV0Z60660521) and the Ministry of Education of the Czech Republic (MSM 6007665801). V. Šlajchrtová and E. Zadáková are greatly acknowledged for field work and laboratory analyses. Mr and Mrs Kamír are thanked for allowing access to the experimental plot and their significant support of the field work.

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Evaluation of Crude Glycerol from Biodiesel Production as a plant pathogen control agent

Diánez F., Santos M.*, de cara M., Tello J.C.

Dpto. Vegetal Production. University of Almería. 04120. Spain

*E-mail: msantos@ual.es

The increase in Biodiesel fuel production is creating the problem of generating a large quantity of glycerine as a subproduct from manufacturing this biofuel. The main objective of this research was to determine glycerine toxicity on seeds as well as the possible doses of application by evaluating germination capacity and root growth in seeds from various plant species (tomato and cucumber). Likewise, the biocidal capacity of glycerine as an alternative in plant pathogen control was evaluated against the following phytopathogenic fungi and bacteria: *Pythium aphanidermatum*, *Botrytis cinerea, Fusarium oxysporum* f. sp. *lycopersici, Fusarium oxysporum* f. sp. *radicis-cucumerinum, Rhizoctonia bataticola, Phytophthora parasitica Sclerotinia sclerotiorum, Clavibacter michiganensis* and *Erwinia persicina*. Results reveal that the use of this subproduct could entail an ecological alternative which would reduce or even eliminate the excessive use of agrochemicals, simultaneously allowing the use and recycling of local resources destined to protect crops.

Keywords biodiesel subproducts; plant disease, glycerine, seed

Introduction

Biodiesel from vegetable oils or animal fats represents a promising alternative to conventional Diesel fuel [5, 7, 9, 11, 13]. It is biodegradable and non-toxic, has low emission profiles and therefore is environmentally beneficial [7]. Continued and increasing use of petroleum will intensify local air pollution and magnify the global warming problems caused by CO_2 [7]. In a particular case, such as the emission of pollutants in the closed environments of underground mines, biodiesel fuel has the potential to reduce the level of pollutants and of potential or probable carcinogens [7].

The search for environment-friendly fuels, the increase in petroleum prices and the reflection on the limitedness of this resource are the main reasons for searching for alternative fuels. Nevertheless, the rise in fuel production is posing the problem of a large quantity of glycerine generated as a by-product in the manufacturing process of this biofuel, ever increasing as a result of production growth objectives. The percentage of biofuel substitution in the EU should rise from 2% at present to 5.75% by the year 2010, according to the Directive 2003/30/CE. Co-product recovery has always been a concern in the economics of the biodiesel process. For each 90 m3 of biodiesel produced from transesterification of vegetable oils, approximately 10 m3 of glycerol are generated [2, 6]. However, crude glycerol derived from biodiesel production possesses very low value because of the impurities. The conventional practice is to recover glycerol by distillation [10]. Although biodiesel fuels are produced chemically and enzymatically, glycerol is essentially generated as the by-product [3, 12]. Glycerol generated is presently applied, for example, as a ingredient of cosmetics, but a further increase in the production of biodiesel fuels would raise the problem of efficiently treating wastes containing glycerol. Others application of glycerol is in soaps and medicines [5]. With the introduction of large volumes of glycerol coming from biodiesel production, it is imperious to find new applications for this chemical, otherwise the economic feasibility of the biodiesel as a renewable fuel is jeopardized.

The main objective of this research was to determine glycerine toxicity on seeds as well as possible application doses by evaluating germination capacity and root growth. Thus, seeds from various plant species (tomato and cucumber) were used to evaluate glycerine as an active fungicidal and bactericidal substance.

Material and methods

Evaluation of fungal and bacterial growth inhibition by glycerine

For growing the various fungal species, isolates were in PDA medium in 9 cm diameter Petri dishes. Fungi were cultured by weekly inoculation for *Phytophthora parasitica*, *Botrytis cinerea*, *Fusarium oxysporum* f.sp. *lycopersici*, *Fusarium oxysporum* f.sp. *radicis-cucumerinum*, and approximately every four days for *Pythium*

aphanidermatum, Rhizoctonia bataticola and *Sclerotinia sclerotiorum.* Bacterial *Erwinia persicina* and *Clavibacter michiganensis* were maintained in KB medium.

To evaluate the suppressor effect of glycerine on fungal and bacterial growth, tests were conducted *in vitro* with dilutions of glycerine in PDA at 2%, 4%, 6%, 8%, 10% 15%, 20% and 50%. Dilutions were made from the glycerine obtained as a co-product of biodiesel fuel, sterilized in an autoclave at 120ºC for 30 minutes (SBG: sterilized biodiesel glycerine) and without sterilization (BG: biodiesel glycerine). Likewise, and in order to compare results, pure sterilized (PSG: pure sterilized glycerol) and unsterilized (PG: pure glycerol) were used. The phytopathogenic fungi for the assays were obtained from the maintenance dishes. With the help of a punch, 5 mm discs in active growth were taken, always from the periphery of the colonies. Each disc was placed in the centre of a Petri dish. The dishes were incubated in the oven in darkness at 25-26ºC for one week with the exception of *Pythium aphanidermatum* and *Sclerotinia sclerotiorum* which were incubated for 3 days. Bacterial isolates were streaked over the culture medium from an isolated colony. Five repetitions were carried out and the results were compared to controls with no glycerine application. Growth was analysed by measuring the diameter of the centre of the disc. Two measurements were taken, i.e. two perpendicular diameters. In the end the average of both values was used as representative data. Lastly, the results were statistically analyzed with the STATGRAPHICS 5.1 (S.G.S., 2001) program to evaluate the efficacy of extract concentrations for fungal suppression.

Determination of glycerine phytotoxicity to seeds

San Pedro tomato seeds and "Marketmore 70" cucumber seeds were used in this study.

Several assays were performed in order to select the best conditions for seed germination: (1) on wet filter paper and (2) between wet filter papers, with different volumes of water. Seeds were washed with 20 ml of sodium hypochlorite solution (0.2 $g \cdot l^{-1}$), then with distilled water (25 ml, three times) and scarified prior to germination in order to avoid fungus proliferation and improve seed germination.

Less fungus proliferation was observed in germination on wet filter paper. From each species 75 seeds were germinated on three filter papers in Petri dishes. The papers were moistened with 3 ml of distilled water or 2%, 4%, 6%, 8%, 10% 15%, 20% and 50% glycerine solutions (SBG, BG, PSG and PG). Wet filter papers with 15 seeds each were distributed in 3 trays. Samples were taken at 0 (control), 1, 2, 3, 4, 5, 6, 7, 8 and 9 germination days. The germination process was repeated twice for each species, and the germination capacity was evaluated by percentage of germination and radicle growth.

Results and discussion

Figure 1 show the results of treating tomato and cucumber seeds with the various concentrations (2, 4, 6, 8, 10, 15, 20 and 50%) and types of glycerine used in the assay (BG, SBG, PG and PSG). High glycerine phytotoxicity existed for both plant species in all treatments. Complete inhibition of tomato and cucumber seed germination started from 2 and 4% respectively, as well as a drastic reduction in radicle length at the same concentrations. Sterilising BG and PG decreased phytotoxicity in tomato, but given the high toxicity observed, this was only detectable at the 2% concentration. A similar situation occurred with cucumber seeds. Several studies have shown that glycerol is toxic to plants and that this toxicity is likely to be caused by glycerol metabolism [1] [4]. Other authors such as Liang et al. [8] have demonstrated that solutions with concentrations greater than 60% were not absorbed by navy bean seeds, and lower concentrations reduced seed germination. Immersion in either 25% PEG or 60% glycerol solutions did not diminish germination although seedling vigour was slightly reduced.

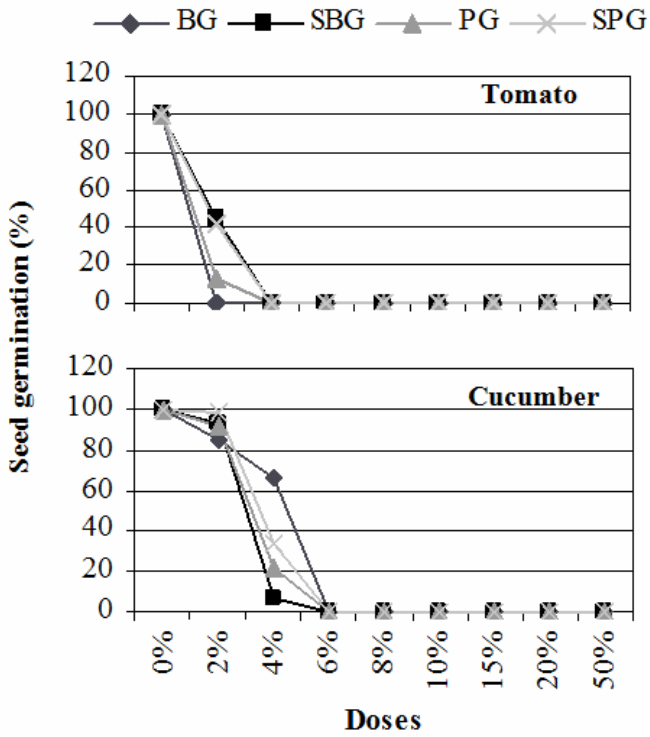

Fig. 1. Glycerine toxicity on seeds. BG. Biodiesel glycerine; SBG sterilized biodiesel glycerine; PG. Pure glycerine; SPG. Sterilized pure biodiesel.

Results indicate that the use of glycerine as a humectant or substrate enhancer for plant development can involve the risk of phytotoxicity. Other application systems should be tested since in this case application was made directly on the seed. Better results might be obtained when applied to the substrate or through irrigation. At any rate, there was no germination promoting effect from the doses and conditions of this assay.

Figure 2 shows the effect of the various treatments on the fungi and bacteria under study. The application of BG in low concentrations had a mycelial growth inhibitor effect on the assayed fungi. Nevertheless, inhibition is only observed at the maximum concentration tested. *Botrytis cinerea* proved to be the most resistant fungi to glycerine application, with no significant reduction of mycelial development at concentrations of 15% or less. Both bacterial species showed diverse performances, with gram-positive *Clavibacter michiganensis* being more susceptible to the various treatments, probably due to the composition of its bacterial wall. Likewise, gram-negative *E. persicina* showed increased colony development from the BG treatment, with inhibition of development at a concentration of 50%. The BG inhibitor effect was increased for all the fungi tested when BG was sterilized, with complete inhibition at concentrations of 8 and 15%. The effect of pure glycerine was not uniform among the various species, and in no case was there a biocidal response at concentrations below 50%. At lower concentrations PG performed as a biostatic. In the case of *E. persicina*, none of the concentrations tested inhibited its development. Significant differences did not exist in results from the application of PG or SPG.

At present, research has not been undertaken on the application of glycerine obtained from the production of biodiesel for plant development or for pathogen control, so results could not be contrasted. Studies are generally on the use of PG as an adjuvant for increasing the efficacy of some pesticides and as mentioned above, to increase the efficacy of antibiotic treatment in seeds.

Given these results, perhaps research should be directed towards controlling weeds and other pathogens. Since Oomycetes were the most susceptible fungi to glycerine application, probably due to the tensoactive effect of glycerine, it would be quite interesting to discover which doses might be effective when applied in irrigation.

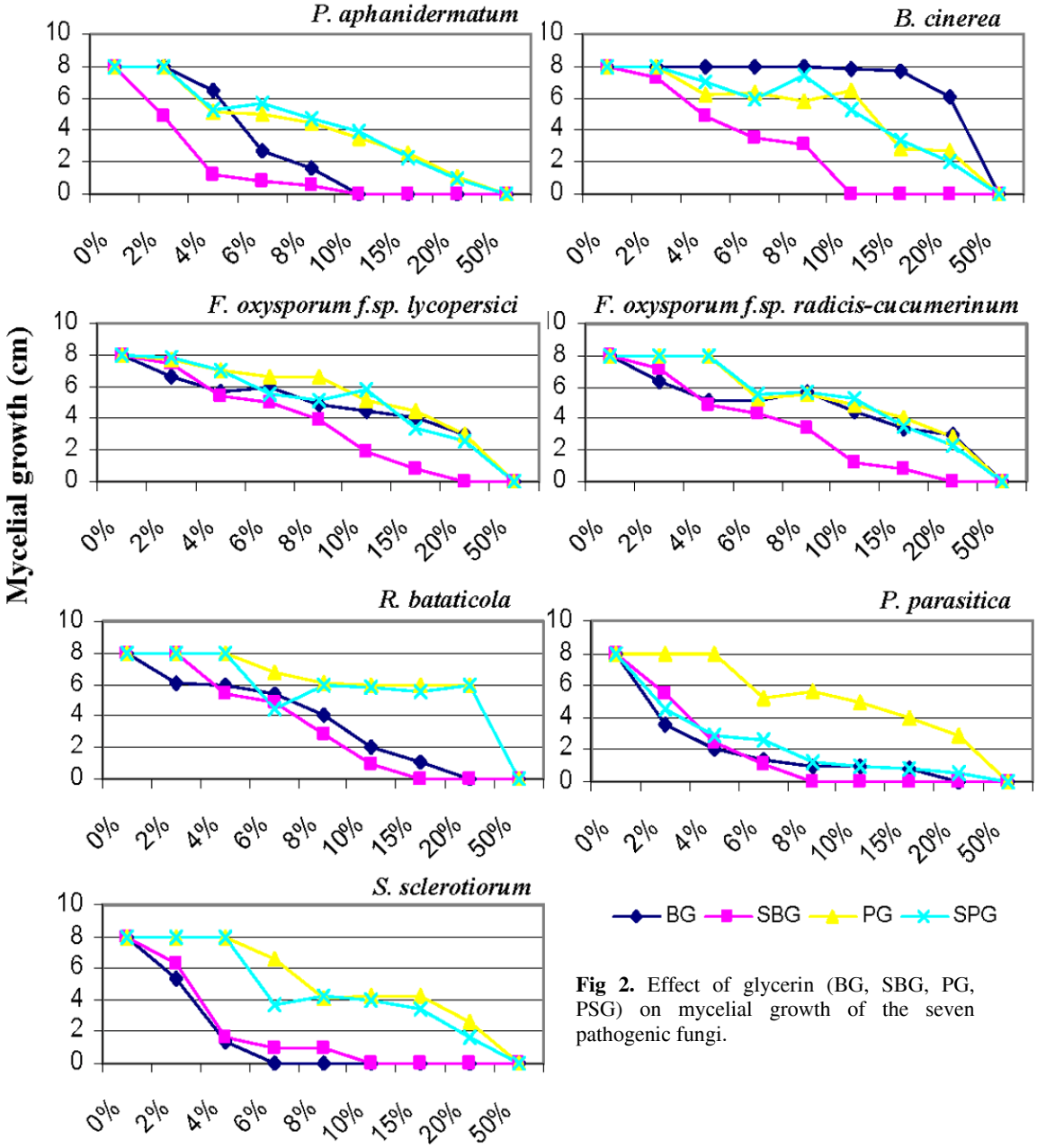

Fig 2. Effect of glycerin (BG, SBG, PG, PSG) on mycelial growth of the seven pathogenic fungi.

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Extended nutrient limitation influences *Ralstonia solanacearum* survival in natural water microcosms

B. Álvarez[1], M. M. López[1], and E. G. Biosca[2]

[1] Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA). Carretera Moncada-Náquera, km 4.5. Moncada, 46113. Valencia, Spain

[2] Departamento de Microbiología y Ecología, Universidad de Valencia. Av. Dr. Moliner, 50. Burjasot, 46100. Valencia, Spain

Ralstonia solanacearum biovar (bv) 2 causes bacterial wilt and potato brown rot in solanaceous plants in temperate areas. The pathogen is able to persist in the environment, where it is frequently disseminated by watercourses. In these habitats, the effect of long-term oligotrophy on its survival remains to be ascertained. On that purpose river water microcosms were inoculated with *R. solanacearum* bv 2, incubated at 24ºC and monitored for total, viable and culturable bacterial cell numbers up to four years. Within the first year, *R. solanacearum* bv 2 populations remained roughly constant in the initial levels. Then and until the fourth year, total counts slowly increased, while viability slightly declined and culturability decreased to a greater extent, pointing out a proportion of the bacterial populations entering the viable but non-culturable state. As cells in this state are not detected by cultivation-based methods, they represent a new challenge in designing strategies for control of the bacterial wilt disease.

Keywords bacterial wilt; water; microcosms; persistence; oligotrophy; culturability; viability; VBNC

1. Introduction

Ralstonia solanacearum (E.F. Smith) is a phytopathogenic water borne bacterium causing severe wilting in numerous crops and diverse ornamental plants of economic interest worldwide [1]. The species is classified into biovars according to biochemical characteristics [2], with biovar (bv) 2 affecting mainly solanaceous plants and being the causative agent of potato brown rot in temperate areas [3]. It is considered as a bioterrorism agent in the U.S.A. [4] and a quarantine organism in the European Union [5]. The bacterium is a vascular pathogen [2, 3], able to survive in the environment in the absence of host plants mainly in water, soil, plant debris and reservoir plants [6]. It persists in water for variable periods [7-9], and waterways frequently disseminate the pathogen favouring outbreaks of the disease [6, 10]. The subsistence of this bacterium in harsh environmental conditions might be somehow related to its ability to enter the viable but non-culturable (VBNC) state [11]. *R. solanacearum* becomes VBNC after exposure to copper [12], and incubation at low temperature in soil and water [9, 13, 14].

In Spain in the last years *R. solanacearum* bv 2 has caused wilting in potato and tomato cultures and it has been isolated from river and irrigation water [15], where it can survive in the presence of water microbiota [16]. The objective of this work was to monitor long-term survival of *R. solanacearum* bv 2 in sterile river water to assess the effect of extended nutrient limitation conditions, characteristic of oligotrophic aquatic habitats.

2. Materials and methods

R. solanacearum bv 2 strain IVIA-1602.1 was used to inoculate the survival microcosms. This strain had been isolated from potatoes with brown rot symptoms in Spain. It was kept at –80 ºC in a 30% (v/v) glycerol medium, and for survival experiments it was cultivated on Yeast Peptone Glucose Agar (YPGA) [17] for 72 h at 29ºC.

River water samples from a Spanish river where the bacterium had previously been detected were collected according to [15]. River water microcosms for survival assays were composed of 200 ml of 0.22 µm filtered and autoclaved river water inoculated with the strain IVIA-1602.1 of *R. solanacearum* bv 2 to a final concentration of $5x10^6$ cfu/ml, similarly to [16]. All the microcosms were done in triplicate and incubated at 24ºC for four years.

During the whole four-year period, aliquots from each river water microcosm were taken to perform total and viable cell counts by microscopy-based methods and culturable cell counts by plating at inoculation time and after one, two, three and six months, and then every six months up to four years. To distinguish total from viable cells, the direct viable count (DVC) method [18] was applied as modified by [9] and it was optimized to 72 h from the first to the fourth year of microcosm incubation. According to the method, after incubation of the

bacterial cells with yeast extract and nalidixic acid, viable cells elongate without dividing (Fig. 1). Visualization of the cells was done at each time after cell fixation by formaldehyde 2% (v/v) and cell staining by acridine orange 0.1% (w/v) as described [19]. Average of total and viable *R. solanacearum* cells were counted in at least 20 random fields with a Leika epifluorescence microscope at an amplification of x1250. For culturable cells, plate counts on YPGA agar were performed after 72 h at 29 ºC.

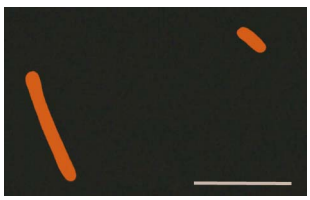

Fig. 1 *Microscopic observation of R. solanacearum bv 2 strain IVIA-1602.1 cells.* After incubation with a nutrient (yeast extract) and a cellular division inhibitor (nalidixic acid), only treatment-responsive elongated cells are considered as viable (on the left), and non-elongated ones as non-viable (on the right). Scale bar: 5 µm.

3. Results

3.1 Effect of nutrient limitation on survival of *R. solanacearum* bv 2 in natural river water microcosms for the first year

In the river water microcosms at 24ºC total, viable and culturable *R. solanacearum* counts remained roughly constant in the population levels observed at inoculation time in samplings at one, two, three and six months, and up to one year after microcosm inoculation. Within the whole period, total cell numbers kept above 10^7 cells/ml with a slight increase from the sixth month, whilst viable cell numbers were nearly below 10^7 cells/ml, and culturable cell counts remained approximately at 10^6 cfu/ml (Fig. 2). Similar results were obtained with the other water samples.

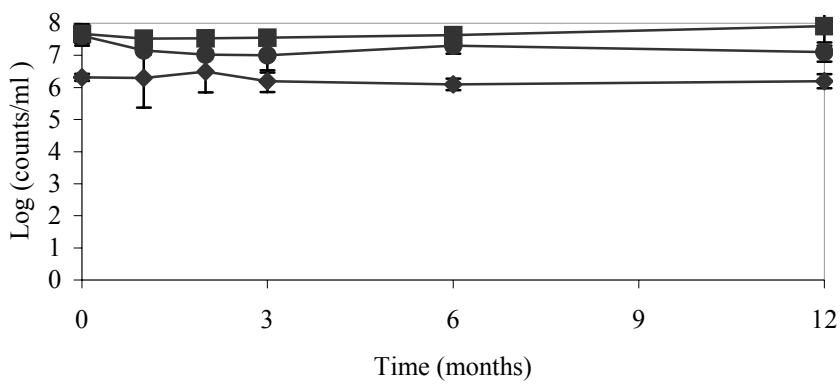

Fig. 2 *Population trends of R. solanacearum strain IVIA-1602.1 in river water microcosms at 24ºC for the first year post-inoculation.* Total (■), and viable (●) cells, and colony forming units on YPGA (♦). Points are the mean of triplicate microcosms and error bars indicate variation as standard deviation for each point.

3.2 Effect of nutrient limitation on survival of *R. solanacearum* bv 2 in natural river water microcosms from the first to the fourth year

In the river water microcosms at 24ºC, after the first and throughout the second, third and fourth years, total *R. solanacearum* counts slowly increased up to 10^8 cells/ml, whilst viability slightly declined to around 10^6 cells/ml, and culturability until about 10^4 cfu/ml (Fig. 3). Differences in population numbers between viable and culturable *R. solanacearum* cells indicated a proportion of the bacterial populations entering the VBNC state. Similar results were obtained with the other water samples.

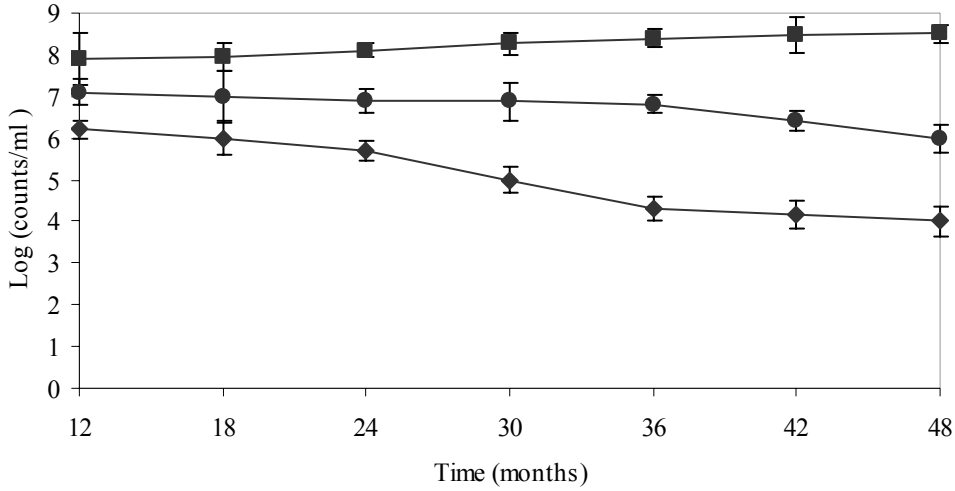

Fig. 3 Population trends of R. solanacearum strain IVIA-1602.1 in river water microcosms at 24ºC from the first to the fourth year post-inoculation. Total (■), and viable (●) cells, and colony forming units on YPGA (♦). Points are the mean of triplicate microcosms and error bars indicate variation as standard deviation for each point.

4. Discussion

Environmental waters are a means of transmission of *R. solanacearum* bv 2 in natural ecosystems, where the pathogen may survive for variable periods until contact with a host plant [6]. Population densities of *R. solanacearum* bv 2 in natural surface waters for as long as three years were recently monitored [15, 20] and correlated with water temperature [15]. Growth temperature together with incident light, sediment, and seawater salts were evaluated in relation to the culturability of the bacterium in environmental water [9], as well as the presence of native microbiota [16]. Notwithstanding scarce data are still available on the effect of prolonged exposure to oligotrophic conditions on the ability for survival of *R. solanacearum* bv 2 in natural water under controlled conditions.

Within the first year of nutrient limitation in sterile river water microcosms at 24ºC *R. solanacearum* bv 2 total, viable and culturable populations remained similar to inoculation levels. That confirmed the persistence of this plant pathogen in nutrient-limited aquatic environments according to previous reports on the culturability of the bacterium at a similar temperature in agricultural drainage water for four months [9], in tap water for around seven months [7] and in river water for one year [8]. Scarcity of nutrients apparently did not interfere with *R. solanacearum* culturability during one year in river water microcosms, as described for *Erwinia amylovora* after six months in irrigation water [21] or *Pseudomonas fluorescens* after one year in agricultural drainage water [22].

Progressively within the second, third and fourth years under nutrient deprivation in sterile river water microcosms at 24ºC diverse trends were diplayed by total, viable and culturable *R. solanacearum* bv 2 populations. A slight increase in total cell numbers was observed suggesting that reductive divisions of *R. solanacearum* bv 2 cells might be taking place in the microcosms, similarly to other bacterial species under long starvation [23]. However, in the same time period, *R. solanacearum* bv 2 viability and culturability decreased, although with different slopes. The distinct viable and culturable population sizes indicated a proportion of starved viable populations becoming non-culturable due to the extended oligotrophic conditions undergone in the river water microcosms. Likewise, other bacteria have entered the VBNC state under prolonged starvation [24, 25]. A great many bacterial cells may be induced into this physiological state as a response to some form of natural, environmental stress, favouring their survival in adverse environmental conditions [25, 26]. Although cells in this state fail to form colonies, they are alive and capable of renewed metabolic activity [11, 25, 26]. VBNC significance seems evident in studies where culturability is employed as indicator of viability [25], with special concern with regards to pathogenic agents.

Overall, this work presents evidence on the high potential for subsistence of *R. solanacearum* bv 2 in energy-deficient systems. The pathogen was able to survive for four years in oligotrophic river water microcosms, indicating that it is an efficient scavenger of scarce nutrients. Within the period, a proportion of the bacterial population became VBNC. As cells in this state are more difficult to be detected by standard protocols, they represent now a new challenge to be overcome in control and management of bacterial wilt disease.

Acknowledgements The support by projects FAIR 5-CT97-3632 and QLK 3-CT-2000-01598 of the European Union and FD 1997-2279 of the Ministerio de Educación y Ciencia of Spain are gratefully acknowledged. B. Álvarez thanks the Instituto Valenciano de Investigaciones Agrarias for a predoctoral grant.

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Genetic characterization of *Plutella xylostella* with resistance to formulates of *Bacillus thuringiensis* at laboratory and field level

J. A. Marroquín-Ortega[1], V. E. Aguirre-Arzola[1], V. R. Moreno-Medina[2], R. Bujanos-Muñiz[3], L. Galán-Wong[1], L. H. Morales-Ramos[1] and B. Pereyra-Alférez[1,*]

[1] Instituto de Biotecnología. Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León. Pedro de Alba y Manuel L. Barragán S/N. Cd. Universitaria. San Nicolás de los Garza, NL. 66450. México
[2] CBG-IPN, Reynosa, Tamps. México
[3] INIFAP-Celaya, México

[*]E. mail: bpereyra@fcb.uanl.mx

Plutella xylostella collected from different broccoli fields, were bioassayed with Xentari™ a commercial formulate based on *Bacillus thuringiensis* (Bt). Results of initial LC50 (mg/L) was of 0.294 Xentari™ and a resistance ratio (RR) (LC50 field colony / LC50 of laboratory colony) of 1.59. We obtained a colony that resisted 200-times the LC50 Xentari™ concentration. This colony had a LC50= 16.43 (FL95= 14.52 - 24.06), 56-fold in comparison with its initial susceptibility and 89-fold than laboratory one. Results of bioassays with solubilized protoxins of Cry1Aa, Cry1Ab and Cry1Ac showed significant differences (LC50, µg/ml) for Cry1Ab. Resistant had a LC50 of 0.025, while susceptible 0.003. The 200X colony had 1.8-fold resistance than laboratory for Cry1Aa. Genetic characterization of 200X and susceptible laboratory colonies was done by amplification of EaaMcta-04.70, a STS associated with resistance to Bt toxins. STS-PCR product was visualized only in the 200X. This molecular marker was positive too for seventeen larvae collected from a field where was observed a reduction of sensitivity to Bt products. This is the first report where the molecular marker EaaMcta-04.70 could be is associated with resistance to *B. thuringiensis* formulates in México.

Keywords *Plutella xylostella*; bioassay; molecular marker; Cry protein resistance

1. Introduction

The common soil bacterium *Bacillus thuringiensis* (Bt) produces crystals containing proteins that are toxic to certain insects, but are harmless to another organisms including people, wildlife, and most beneficial insects. Genes encoding Bt toxins have been incorporated into and expressed by crop plants, thus providing environmentally benign control of insects pest. Lepidopteran larvae, which include some of the world's most damaging crop pests, are the primary targets of more than 99 % of the acreage of currently deployed, Bt-producing transgenic plants [6].

Evolution of resistance by pests is a serious threat to the continued efficacy of Bt toxins. Although the diamondback moth (*Plutella xylostella*) is the one of some insect with resistance to Bt toxins in open-field populations, laboratory selection has produced resistance in several order species of Lepidoptera. With millions of hectares of Bt toxin-producing transgenic plants grown yearly, other pests are likely to evolve resistance quickly unless effective countermeasures are designed and implemented soon. Better understanding of the transgenic basis of resistance is essential for developing such countermeasures.

In our country, Guanajuato is one of most important agricultural estates of México, especially in broccoli and potato production. These fields have been sprayed with Bt products for more than 10 years, and in some of them was observed a reduced sensibility of *P. xylostella* to Bt formulates, mainly Xentari™ and Dipel™. In this work we analyze the some biochemical and genetic items in resistant and sensible *P. xylostella* colonies, and describe the genetic amplification of the STS EaaMcta-04.70, which was found only in resistant colonies, but not in sensible diamondback moth nor *Trichoplusia ni*.

2. Material and Methods

2.1. Bioassay: Insects were collected from two different fields of broccoli (Here North and East zone) located at State of Guanajuato. Several larvae (15) were washed and stored separately at -20°C. Other ones were placed on broccoli leaves until adult stage. The F1 was obtained and bioassayed with Xentari™ and Javelin™. Survivors were under selective pressure with Xentari™ until the formulate concentration was of 200X the initial LC50.

2.2. DNA Purification: Third instar larvae were choosing and washed separately with sterile double-distilled water and placed on microcentrifuge 1.5 ml tube. In order to avoid contamination, new material was used for each larva. One ml of extraction buffer (Tris-HCl 50 mM, pH 8.0, SDS 2 %, NaCl 0.75 M, EDTA 10 mM) was

added and mashed. Then 0.5 ml of proteinase K (Sigma) was added and mixtures incubated at 65°C for 30 min. The tubes were centrifuged and supernatant transferred to another clean and sterile tube. DNA was extracted with chloroform (one time) and phenol-Choloroformo one time and upper phase was taken and transferred to another 1.5 ml tube. DNA was precipitated with ethanol, dried and resuspended with 100 µl of water [10].

2.3. PCR Conditions: Genetic amplifications were carried out essentially as mentioned elsewhere using the sequences called as c39-451p1 and c39-451p2 [5]. Reaction mixtures were composed as follows: buffer/$MgCl_2$ 10 X; dNTPs, 200 µM; primers, 100 nM; DNA, 10 ng; Taq DNA polymerase (Promega) 2.5 U; final volume of 50 µl. Thermocycler conditions on the Perkin-Elmer DNA thermocycler 2400 were: 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final 10 min at 72°C. PCR products were separated on agarose 1.5 % gel and stained with ethidium bromide.

2.4. STS Cloning: The PCR fragments were cloned into a DNA TOPO TA cloning system pCR 4.0 (Invitrogene) and the transformations were carried out in *E. coli* TOP10™ strain as recommended by manufacturer (Invitrogene). The presence of the 320 bp fragment was verified with PCR.

3. Results

3.1 Bioassay:

Results of initial LC50 (mg/L) was of 0.294 Xentari™ and a resistance ratio (RR) (LC50 field colony / LC50 of laboratory colony) of 1.59. Both colonies were under selection pressure with Dipel™, Javelin™ and Xentari™ formulates, but we obtained resistance only for Xentari™ and only for the North Zone colony. We obtained a colony that resisted 200-times Xentari™ concentration. This colony had a LC50= 16.43 (FL95= 14.52 - 24.06), 56-fold in comparison with its initial susceptibility and 89-fold than laboratory one (Table 1).

Table 1. LC_{50} of colonies under selection pressure with Bt formulate

Colony	Product	LC50 (mg/l)	Slope	Confidence Interval (95%)
Susceptible	Xentari™	0.184	1.893	0.138-0.237
North Zone	Xentari™	16.43	1.820	14.527-24.06
East Zone	Xentari™	0.235	1.564	0.196-0.279

Results of bioassays with solubilized protoxins of Cry1Aa, Cry1Ab and Cry1Ac showed significant differences (LC50, µg/ml) for Cry1Ab. Resistant had a LC50 of 0.025, while susceptible 0.003. The 200X colony had 1.8-fold resistance than laboratory for Cry1Aa (Table 2).

Table 2. Susceptibility of resistant colony to Cry1A toxins

Colony	Toxin	LC50 (mg/l)	Slope	Confidence interval (95%)
Susceptible	Cry1Aa	1.956	1.908	1.483-2.514
	Cry1Ab	0.003	1.338	0.002-0.004
North Zone	Cry1Aa	3.51	1.606	2.366-4.478
	Cry1Ab	0.025	1.312	0.019-0.036
	Cry1Ac	0.46		0.272-0.841
East Zone	Cry1Ab	0.005	1.657	0-003-0.012

3.2 DNA Purification and PCR:

The DNA technique extraction was standardized with sensible larvae of *Spodoptera exigua*. After that, we obtained DNA from *P. xylostella* and *T. ni* experimental and field colonies. DNA was extracted from individual larvae in separately tubes. By means of the PCR technique we identify the genetic marker EaaMcta-04.70 in sensible and resistant laboratory larvae and in resistant ones collected from broccoli fields. Figure 1 depicts the PCR results for the STS EaaMcta-04.70, where we can see that a 320 bp DNA band is present only in *P. xylostella* laboratory resistant (Fig. 1A, lane 4), but not in another lepidopteran insects. When this assay was

extended to other colonies, again, the 320 bp DNA band was positive only for *P. xylostella* in both laboratory and field resistant (Fig. 1 B, lanes 2, 5-9 and 12).

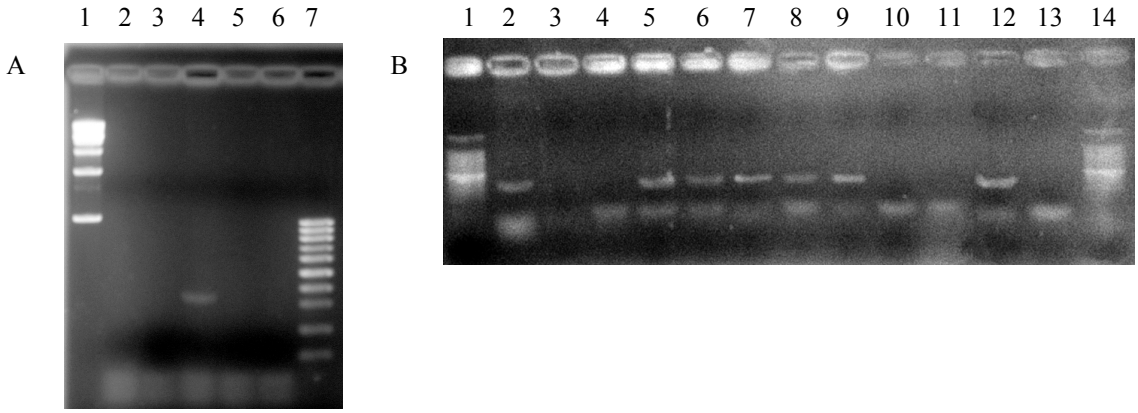

Figure 1. Presence of the STS fragment in sensible and laboratory and field resistant colonies. Panel A. Lanes: 1, Ladder 1 kbp; 2, *S. exigua* laboratory resistant; 3, *T. ni*; 4, *P. xylostella*, field resistant; 5-6, *P. xylostella*, sensible; 7, Ladder 100 bp. Panel B. Lanes: 1, Ladder 100 bp; 2, *P. xylostella* field resistant, 3, negative control; 4, *T. ni* laboratory resistant; 5-9, *P. xylostella* laboratory-resistant; 10, *P. xylostella* sensible; 11-12, *P. xylostella* from broccoli fields; 13, Negative control; 14 Ladder 100 bp.

3.3 STS Cloning:

PCR product from different colonies were cloned in *E. coli* and sequenced. Different *E. coli* recombinant colonies were picking up and seeded on new Petri dishes with antibiotic and the genetic EaaMcta-04.70 marker was searched. We selected eleven different clones. Plasmid DNA was obtained and PCR performed as mentioned before. The presence of the 320 bp band was detected in all recombinant colonies (Fig. 3). DNA from six different colonies was sequenced and nucleotide sequence analyzed and compared with original (Genbank access: DQ993349).

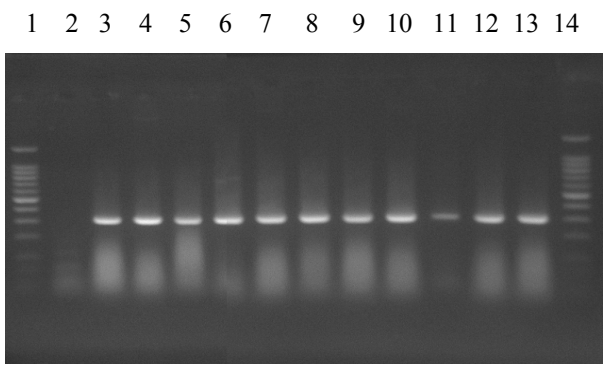

Figure 2. PCR of DNA from recombinant *E. coli* carrying the 320 bp fragment. Lanes: 1 and 14, Ladder 100 bp; 2, negative control; 3-13, PCR products from recombinant clones.

4. Discussion

The larvae used in this project were collected from two different broccoli fields from Guanajuato. The bioassay of the 2 localities, demonstrated to susceptibility to formulated commercial the Xentari™ and Javelin™ finding a LC50 of 0,279 and 1,913 respectively; with the treatment of selective pressure with formulated the commercial Xentari™ a LC50 of 16,43 was obtained (FL95=14.52-24.06), 56 times more compared with the initial susceptibility and 89 times more compared with the one of laboratory, these results demonstrate a lost one of susceptibility to the commercial formulated ones [3] to laboratory level [9]. The putative develop of resistance could be increased with the constant application of formulated Bt to *P. xylostella* in laboratory and in field [1-4, 8, 9, 11].

In the PCR we found a fragment of 320 bp corresponding to the genetic marker EaaMcta-4.70 in larvae of *P. xylostella.* The results revealed the amplification fragment only in resistant *P. xylostella* larvae as it was demonstrated in previous studies [5]. It is possible to emphasize that, the fragment obtained in the present work, was amplified in resistant larvae types, laboratory and field. This is the first report where the genetic marker EaaMcta-4.70, is found in larvae different than reported by Heckel et al [5]. The finding of the fragment in our results indicates that the genetic marker might be used to check the resistance of *P. xylostella* at field level.

Acknowledgements This work was supported by CONACYT grants 38040-N and U46730-Z. JA Marroquín-Ortega was recipient of a fellow from CONACYT.

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Hemicelluloses: from wood to the fermenter

M.G.V.S. Carvalho[1], C.F.A. Baptista[1], C.V.T. Mendes[2] and J.M.S. Rocha[1,*]

[1] – CIEPQPF, Dep. Chemical Engineering, University of Coimbra, R. Sílvio Lima, Pólo II, 3030-790 Coimbra, Portugal

[2] – RAIZ – Forest and Paper Research Institute, Qt[a]. de São Francisco, Ap. 15, 3801-501, Eixo, Portugal

[*]Corresponding author: e-mail: jrocha@eq.uc.pt, Fax: +351239798703

The valorisation of hemicelluloses, as a subproduct of the pulping process, can be achieved with extraction of wood chips by primary hydrolysis (auto or acid catalysed) before cooking, where part of them are dissolved. However, the extracts of the hardwood used in this work (*Eucalyptus globulus*) could contain oligomers, mainly xylose based, that must be further hydrolysed to become a raw material for fermentation to produce *e.g.* bioethanol. The primary extract from an acid hydrolysis can be directly metabolised by microorganisms being a secondary hydrolysis worthless. On the other hand, twice the monosaccharides concentration was obtained when a secondary hydrolysis was performed, over the extracts obtained from the primary auto-hydrolysis. The best conditions for that reaction include 3 h of hydrolysis, with 4 %(w/w) of H_2SO_4 and the highest concentration of solids (*i.e.* avoiding the dilution of primary extracts), which led to a secondary hydrolysis yield as high as 84 %.

Keywords hemicelluloses, xylan, hydrolysis, xylose

1. Introduction

The permanent expectation of a new oil crisis, associated to political instability of suppliers or increasing demand from developing countries, as well as the strong environmental impact of burning fossil resources, with the green-house effects and climate changes, stress the need to reduce the weight of the traditional refinery in the modern economy. Nowadays, there is a great concern about the ecological and energetic valorisation of the forest, the wood residues and their derivatives. The biorefinery concept, providing chemicals and energy from renewable resources, is the way to keep a high level of life quality and environmental sustainability, in a time of irreversible shortage of fossil resources. The pulp and paper industry is the largest scale activity that already uses renewable raw materials, taking advantage of wood cellulose to produce paper, which is a symbol of modern life. However, other wood components, such as hemicelluloses that represent about 25 %(w/w) of the raw material, have been partly wasted during wood chips cooking. The major pulping process, the kraft cooking, uses a mixture of sodium hydroxide and sodium sulphide in an aqueous solution, the so-called white liquor. The reaction occurs in a very alkaline reaction medium under hard process conditions, such as high temperatures (140-170 ºC), high pressures (7 – 12 bar) and long reaction times (1 – 3 h). In fact, part of the hemicelluloses is dissolved in the resulting black liquor, together with lignin, being burnt in the recovery boiler to produce electricity and thermal energy. Having lower calorific content than lignin, hemicelluloses could preferably be extracted from wood, prior to cooking, to generate more value-added products.

To valorise this pulp process by-product, an additional unit operation for wood chips pre-treatment could be included to extract hemicelluloses prior to cooking and produce value-added compounds, *e.g.* bioethanol [1]. Apart from a biodegradation process that could also be used, the previous chemical extraction of hemicelluloses is designated here by primary hydrolysis of wood chips. This extraction can be driven by the action of temperature (primary auto-hydrolysis), or catalysed by an inorganic acid (primary acid hydrolysis). Whatever the methodology, both primary extracted hemicelluloses could still be oligomers that must be hydrolysed to monomers (such as glucose or xylose) before they can be used as carbon and energy source in a fermentation process catalysed by suitable microorganisms, *e.g.* for bioethanol production.

The secondary hydrolysis can be chemically catalysed and the study of the best conditions to improve its yield, that is, to obtain the highest concentration of monosaccharides, was the aim of this study. Coming from a hardwood (*Eucalyptus globulus*), the primary extracts are rich in xylan and the resulting monomers are essentially xylose. Therefore, the scope of this work is the valorisation of hemicelluloses that, otherwise, would be partly dissolved during the cooking process. However, the acquired knowledge can be extrapolated to a more comprehensive biorefinery concept, where all the wood biomass can be available to be converted to fermentable sugars.

2. Materials and Methods

2.1 Primary hydrolysis (of wood)

Two sets of samples were obtained from the treatment of *Eucalyptus globulus* wood chips. The first one resulted from an auto-hydrolysis induced at 150ºC during 180 min in water. The liquid extracts had an average solid concentration of 30 g/L and an average xylose equivalent concentration of 9.4 g/L. The second set of samples came from the acid hydrolysis of wood chips using 0.4% (w/w) sulphuric acid at 140ºC during 180 min. The liquid extracts obtained had an average solid concentration of 40 g/L and an average xylose equivalent concentration of 40.2 g/L.

2.2 Secondary hydrolysis (of oligosaccharides)

The liquid extracts from the primary hydrolysis were diluted (or not) and a 50% (w/w) sulphuric acid solution was added to achieve the desired acid concentration in a final sample volume of 50 mL. These solutions were heated in distillation flasks using heating mantles under reflux conditions at atmospheric pressure. A set of experiments following a 2^3 factorial design [2] was performed using two levels (low and high) of the three selected variables: the acid concentration, the content of dissolved solids and the time of hydrolysis reaction (Table 1). A further and more restrict set of experiments were carried out to better evaluate the effect of the main variables.

2.3 Xylose evaluation

HPLC analysis [3] of liquid extracts showed that xylose was the most abundant sugar, particularly for the acid hydrolysis. For routine analysis another method was followed to determine xylose concentration, before and after the secondary hydrolysis. Each sample was diluted and neutralized with 10% (w/w) sodium hydroxide solution. The samples were then submitted to the DNS (di-nitro salicylic acid) colorimetric method for reducing sugars evaluation, as equivalents of xylose, through the use of previously prepared calibration curves. For this purpose, analytical grade xylose from Merck was used to prepare aqueous solutions with concentrations between 0.1 and 1 g/L and optical densities were below 0.6, read at 540 nm, using a UV/visible spectrophotometer, model Beckman D.U. 650. The secondary hydrolysis yields were calculated by the ratio of xylose liberated in the secondary hydrolysis to the xylose already obtained in the first hydrolysis, to evaluate the need or the success of this second step. At least two repetitions of the assays were carried out to confirm the results and to determine the effect of each variable and cross effects of variables, taking the yield as a dependent variable.

3. Results and Discussion

A secondary hydrolysis was performed on the liquid extracts obtained from the treatment of eucalyptus wood chips with aqueous solutions (auto-hydrolysis or hydrolysis catalysed by an inorganic acid). The aim of this second reaction is to hydrolyse the dissolved hemicelluloses and, consequently, to increase the content of monosaccharides to be used as raw material (carbon and energy source) in ethanol fermentation [3]. Eucalyptus is a hardwood whose main hemicelluloses are polymers of xylose. Being xylose a reducing sugar, the total content of reducing sugars, before and after the secondary hydrolysis, was evaluated by the DNS colorimetric method, as equivalents of xylose, by using calibration curves. These values were used to calculate the yield of monosaccharides production. The final content of xylose equivalents can be compared to the concentration of the dissolved solids in the primary extracts (evaluated by evaporation and weighting), also to check the specificity of the primary extraction (wood chips hydrolysis) on hemicelluloses. At the end of the reaction, and because the mixture was acidic, samples were neutralized with NaOH to avoid interference with the DNS quantification method. However, no interference was observed when neutralized samples were compared to non neutralized ones and, consequently, both assays were used as replications of the analytical experiments. An average of the replications of the xylose concentration is presented.

The hydrolysis of dissolved hemicelluloses was chemically catalysed by sulphuric acid under reflux conditions. Three main variables were identified to be the most important ones concerning the kinetic and the hydrolysis yield: the concentration of H_2SO_4 (% w/w), x_1, the concentration of dissolved solids (g/L), x_2 and the reaction time (h), x_3. A factorial design with two levels for these three variables, *i.e.* 2^3, gave a total of eight experiments for each wood extract.

3.1 Extracts from auto-hydrolysis of wood

In the secondary hydrolysis of the liquid extracts obtained from the auto-hydrolysis of eucalyptus wood chips, lower and higher values of each variable were 2 and 4 %(w/w) for x_1, 15 and 27 g/L for x_2 and 1 and 3 h for x_3. The initial concentration of dissolved solids (30 g/L) was recalculated due to dilution after addition of sulphuric acid solution to adjust the desired acid concentration. Table 1 shows the advantage of the secondary hydrolysis in increasing the content of reducing sugars, with a yield that reached *c.a.* 84%. This almost means a doubling of fermentable sugars, thus improving the potential of wood extracts. However, the possible presence of fermentation inhibitors would imply additional separation costs. It can also be observed that auto-hydrolysis is not selective because it does not only extract wood carbohydrates. In fact, just 1/3 of the total extracted solids are directly fermentable sugars and only half of them are carbohydrates, as confirmed by the xylose equivalent concentration measured after the second hydrolysis. Other solids should be separated, *e.g.* by precipitation, before fermentation. Auto-hydrolysis is, however, a soft extraction process that deserves attention mainly because of the expectations of small negative impact on the quality of the pulp.

Table 1 Secondary hydrolysis (acid catalysed) applied to liquid extracts from wood chips auto-hydrolysis: experimental conditions and results.

x_1 [H_2SO_4] (% w/w)	x_2 [solids] (g/L)	x_3 t (h)	[xylose] 1st hyd (g/L)	[xylose] 2nd hyd (g/L)	hydrolysis yield (%)
2	15	1	4.7	7.0	48.9
4	15	1	4.7	8.1	72.3
2	27	1	8.5	12.3	44.7
4	27	1	8.5	14.5	70.6
2	15	3	4.7	7.7	63.8
4	15	3	4.7	8.3	76.6
2	27	3	8.5	13.4	57.7
4	27	3	8.5	15.6	83.5

The experimental plan presented in Table 1 makes evidence that 4%(w/w) sulphuric acid concentration in the reaction mixture led to better results than 2%(w/w). Also, the time of reaction had a positive effect when it was increased from 1 to 3 h. On the other hand, for the remaining variable under study (the concentration of dissolved solids) the results showed that extracts must not be diluted before being submitted to the second hydrolysis. It is evident that the best conditions found in this set of experiments include the use of liquid extracts without dilution (except the required acid for catalysis), a concentration of H_2SO_4 of 4% (w/w) and 3 h of reaction under reflux operation.

The statistical treatment of the 2^3 factorial design of experiments [2] shown in Table 1, using normalized values of each variable, -1 for the lower value and +1 for the higher value, provided the following model that reproduces well the experimental yield values:

$$\text{yield (\%)} = 64.76 + 10.99x_1 - 0.64x_2 + 5.64x_3 - 1.34x_1x_3 + 1.94x_1x_2 + 0.84x_2x_3 + 1.32\, x_1\, x_2\, x_3$$

From this model it can be confirmed that the main influence on the yield comes from the sulphuric acid (variable x_1), with the highest positive coefficient, followed by the reaction time (variable x_3). The concentration of solids (variable x_2) is not important, and the cross effects of variables are not significant as well.

To confirm the effect of each variable, other sets of experiments were performed, keeping constant the value of the remaining variables. Yields presented in Table 2 confirm that dilution of liquid extracts coming from the auto-hydrolysis of wood is not favourable, except for low values of the other two variables, the acid concentration and the reaction time. Taking into account that the dilution of fermentable sugars does not favour the fermentation process, extracts must not be diluted and hydrolysis yield must be controlled by the other two variables. In addition, the reaction time must not be greater than 3 h as shown in Table 3, also because of the energy consumed.

Table 2 Yields of secondary hydrolysis as a function of the dilution degree of extracts, for fixed values of the other two variables.

Dilution	1/1	1/2	1/10	Constant variables
Hydrolysis yield (%)	45	49	67	$[H_2SO_4]$ = 2% (w/w); t = 1 h
	58	64	56	$[H_2SO_4]$ = 2% (w/w); t = 3 h
	71	72	67	$[H_2SO_4]$ = 4% (w/w); t = 1 h
	84	77	33	$[H_2SO_4]$ = 4% (w/w); t = 3 h

Table 3 Yields of secondary hydrolysis as a function of reaction time (t); $[H_2SO_4]$ = 4%(w/w) and [solids] = 27 g/L.

t (h)	0.5	1	3	4
Hydrolysis yield (%)	50	71	84	40

The effect of sulphuric acid concentration was studied for 2, 4, 6 and 9 %(w/w) with non-diluted extracts ([solids] = 27 g/L) and 1 h of reaction. Although better results were achieved when 4% (w/w) was used instead of 2%, as shown in Table 1, no advantages were observed with increasing concentration beyond 6%. In fact, hydrolysis yields of 70% and 44% were obtained for 6 and 9%, respectively. Although lower than the best value presented in Table 1, the use of 4 to 6 % (w/w) of H_2SO_4 and t = 1 h (yield of 70%) allows a significant reduction of the reaction time, important at industrial level to minimize the energy costs associated.

3.2 Extracts from acid hydrolysis of wood

Liquid extracts coming from a primary acid hydrolysis of wood chips were treated by the same process as the ones obtained from auto-hydrolysis. However, it was observed that the reducing sugar content of the extracts from the primary hydrolysis was already much higher than the equivalent extracts coming from auto-hydrolysis. Additionally, the secondary hydrolysis proved to be worthless, not improving the monosaccharides content, as shown in Table 4. The secondary hydrolysis yields were even negative, showing that a degradation of the reducing sugars already present in the primary extracts had occurred. Longer hydrolysis of carbohydrates can even reduce the nutritive value of the extracts to be used as culture media. Another observation from Table 4 is that the concentration of dissolved solids in liquid extracts is similar to the concentration of reducing sugars obtained after the first or the second hydrolysis. This means that the acid hydrolysis of wood chips is very specific to carbohydrates, without extracting other solids in a high amount. From the point of view of obtaining an enriched culture medium for fermentation, this last strategy seems to be favourable and it is the subject of other study [3]. However, within the biorefinery concept application to pulp and paper industry, the main restriction is the quality of the pulp obtained. The primary hydrolysis of wood chips, when acid-catalysed, probably degrades the remaining polysaccharides at higher extent than the auto hydrolysis does. This last and softer wood hydrolysis process may be selected to keep pulp quality, but a secondary hydrolysis will be essential to convert extracts into fermentable compounds.

Table 4 Secondary hydrolysis (acid catalysed) applied to liquid extracts from wood chips acid hydrolysis: experimental conditions and results.

x_1 $[H_2SO_4]$ (% w/w)	x_2 [solids] (g/L)	x_3 t (h)	[xylose] 1st hyd (g/L)	[xylose] 2nd hyd (g/L)
2	20	1	20.1	20.2
4	20	1	20.1	20.1
2	36	1	36.1	33.1
4	36	1	36.1	35.5
2	20	3	20.1	20.1
4	20	3	20.1	19.5
2	36	3	36.1	33.5
4	36	3	36.1	34.6

4. Conclusions

Eucalyptus globulus is a fast growing hardwood and is recognized as a very good raw material for pulp and paper industry. In the cooking process part of the hemicelluloses (mainly xylan) are lost. They can be previously extracted by an auto-hydrolysis and produce added value compounds, *e.g.* bioethanol. Nevertheless, the content of fermentable sugars must be increased by further hydrolysis of extracted hemicelluloses, before they can be used for fermentation. In this chemically catalysed reaction, the concentration of sulphuric acid and the time of reaction under reflux conditions were found to be the most important factors that affect the yield of monosaccharides. A catalyst concentration of 4% (w/w) of H_2SO_4 and 3 h of reaction almost double the fermentable sugars concentration, with a yield of 84%. However, a faster and more economic hydrolysis can be achieved in 1 h using a H_2SO_4 concentration of 4 or 6% (w/w), although with a decrease of the yield to 70%. If an acid-catalysed primary hydrolysis is applied to wood chips, the extracted hemicelluloses become mostly hydrolysed into monosaccharides but, the remaining polysaccharides may also be partly degraded, thus affecting the quality of the pulp.

Acknowledgments: To RAIZ, the supply of the hemicelluloses extracts.

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Identification and characterization of a Cry7-like protein of *Bacillus thuringiensis* GM-33 strain holotype for subsp. *monterrey*

V.E. Aguirre-Arzola[1], A. G, Alcazar-Pizaña[1], L. J. Galán-Wong[1], H. A. Luna-Olvera[1], B.E. Rivera Chavira[2] and B. Pereyra-Alférez[1,*]

[1] Instituto de Biotecnología. Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León. Pedro de Alba y Manuel L. Barragán S/N. Cd. Universitaria. San Nicolás de los Garza, NL. 66450. México

[2] Facultad de Ciencias Químicas. Universidad Autónoma de Chihuahua. Av. Universidad S/N. Cd. Universitaria. Chihuahua, Apdo. Postal 669. c.p.31170, México.

[*]Corresponding author: bpereyra@fcb.uanl.mx

The mexican *Bacillus thuringiensis* strain, GM-33 the holotype strains for the serovar monterrey was characterized in terms of insecticidal activity, putative *cry* gene content and immunological relationship of their crystal proteins with several known insecticidal crystal proteins. Insecticidal crystal proteins from GM-33 displayed marginal toxicity against *Heliothis virescens* and *Trichoplusia ni*. The *cry* gene content was analyzed by PCR method with general primers to detect *cry*1, *cry*2, *cry*3, *cry*4, *cry*5, *cry*7, *cry*8, *cry*9, *cry*11, *cry*12, *cry*13, *cry*14, *cry*21, and *cyt* genes. PCR product was detectable only with primers for the *cry*7A gene. The N-terminal amino acid sequences of trypsin-resistant fragments showed three peptides of about fifteen residues each one, and only one displayed high homology whit Cry7Aa. The 1.9 kbp-PCR product show high homology against the *cry*7 genes reported. Our results of DNA sequence suggest us that GM-33 harbors a gene encoding for a Cry7 protein, however toxicity and partial amino acid sequence addressed for GM-33 could to synthesize a novel Cry protein.

Keywords Cry7, GM33, *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis consist of a great number of isolates from around the world, which display toxicity against a number of insect pests of agronomical, forest and public health importance. The entomopathogenic activity of this bacterium depends for a large part on the production of a crystalline parasporal inclusion which may be comprised of one or more proteins known as insecticidal crystal proteins (ICP), named Cry and Cyt proteins, or more generally, δ-endotoxins [1].

Screening programs to isolate *B. thuringiensis* strains from diverse origins, such as soil, dead insects, plant surfaces, stored grains and fresh water or marine environments revealed that it is a ubiquitous bacterium. Crystal proteins have a large range of specificities against species of different insect orders (Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, and Mallophaga), Acari and other invertebrate organisms, but also some have no known biological activity [2]. *B. thuringiensis* strains and their genes coding for ICPs have been identified and classified on basis of their cry gene sequences and flagellar antigens (antigen H). To date, the nucleotide and deduced amino acid sequences of more than 300 cry genes have been compared and classified in 51 groups and more subgroups (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html) [1]. The classification of *B. thuringiensis* strains based on H-antigens, shows a high variability in the strains isolated around the world [1, 2, 4, 6]. Sixty-nine serotypes and 13 sub-antigenic groups have been identified, giving 82 serovars among the 3500 isolates deposited in the International Entomopathogenic Bacillus Center (Institut Pasteur) collection [3]. The strain, named GM-33, was isolated by our research group and classified as holotype strain for H-28a28b serovar *monterrey*, [3]. In spite of intensive screening programs and development of polymerase chain reaction (PCR) strategies to identify putative *cry* genes [2, 4, 6], many *B. thuringiensis* strains remain to be characterized at serotype and/or biological activity level. In this work, we report the characterization of the ICP from the Mexican serotype strain in terms of their biological activity, putative cry gene content, and crystal protein profile.

2. Material and methods

2.1 *B. thuringiensis* strains: Typical proteinaceous crystal present in GM-33 strain was identified by phase-contrast microscopy. The *B. thuringiensis* strain was classified by the Institute Pasteur (Paris) and it was recognized as holotype for new the serotype H-28a28b serovar *monterrey* [3]. The *B. thuringiensis* strains HD-1, HD-73 (positive control for lepidopteran bioassays), and HD-511 used as reference in this study were obtained from our own collection. The *B. thuringiensis* var. *tenebrionis* (strain 4AA1), was obtained from the

Bacillus Genetic Stock Center, Ohio State University, and was used as positive control in the coleopteran bioassays. All strains were maintained on nutrient agar medium (Difco).

2.2 Spore-Crystal mixtures: A single colony was seeded on nutrient medium agar, 5 petri dishes per strain and incubated for 3-4 days at 30 °C. Spore-crystal mixture were harvested, and washed twice with sterile bidistilled water. Protein concentrations were determined with the Lowry method [5].

2.3 Insect bioassays: Insecticidal activity of *B. thuringiensis* spore/crystal-mixtures (50 and 100 µg of total protein) was tested against several Lepidopteran (*Heliothis virescens*, *Trichoplusia ni* and *Spodoptera exigua*), and Coleopteran (*Leptinotarsa decemlineata*) pests. Biological activity against lepidopteran was carried out with neonate larvae as reported by Bravo et al [2]. Bioassays with coleopteran were done as follows: Six potato leave circles were dipped in a suspension of spore-crystal mixture and allowed to dry. Larvae (12 per circle) of *L. decemlineata* were placed on leaves and incubated. All bioassays were done in triplicate and repeated on three different days. Mortality percentage was recorded after 7 days and 76 h for lepidopteran and coleopteran larvae, respectively.

2.4 Identification of *cry* genes: *B. thuringiensis* strains were grown on nutrient agar for 12 h at 30°C. A loop was transferred to 100 µl of sterile water, and placed in a boiling water bath for 10 min. Cell lysates were centrifuged at 12,000 rpm for 10s (Eppendorf model 5415C centrifuge), and 35 µl of supernatant were used as DNA sample for PCR experiments. In order to detect *cry*1 genes, we used the primers reported by Cerón et al [6] and Bravo et al [2]; for *cry*2 genes we used primers designed by Ibarra et al [7]. While the primers Un3(d)-Un3(r) and Un7,8(d)-Un7,8(r) were used to amplify the *cry*3, *cry*7 and *cry*8 genes, respectively [4]. In the other hand, the genes *cry*9A, *cry*11, *cry*13, nematocidal and *cyt* genes were searched with the primers reported early [2]. PCR mixtures and conditions were carried out as reported by Ben-Dov et al [4]. DNA amplifications were carried out in a DNA thermal cycler (Perkin-Elmer model 2400). PCRs products were separated by electrophoresis on 2% agarose as reported earlier [12].

2.5 Cloning and partial characterization of PCR products: PCR products were purified by using Wizard® PCR preps DNA purification system (Promega), and cloned in *Escherichia coli* with pGEM-T easy vector II (Promega) following suppliers indications. Putative positive clones were selected subject to another PCR with specific primers. Nucleotide sequence from three different colonies was performed in a capillary sequencer (ABI PRISM® 310™. Applied Biosystems). Sequence was compared using basic BLAST (April 2007. http://www.ncbi.nlm.nih.gov/BLAST/). Base on the sequence obtained, a new primer sets were design, from the new sequence to the transcriptional terminator.

2.6 Crystal protein analysis and ICP digestion: Protein profiles of the crystals were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Spore-crystal mixtures (2-3 micrograms) were dissolved in sample buffer and separated on 10 % polyacrylamide gels. SDS-PAGE conditions were performed as mentioned elsewhere [8].

2.7 N-terminal protein sequencing: Proteins were separated on SDS-PAGE as mentioned before. Gels were treated in two ways, one gel was blotted onto nitrocellulose while another band of ca. 130 kDa was elicited, purified and digested with trypsin. Peptides were separated using a 2.1 mm C-18 column on a Michrom MAGIC 2002 HPLC. The N-terminal amino acid sequences of trypsin-resistant fragments were performed automated Edman degradation using a Procise 494 (Applied Biosystems).

3. Results

3.1 *B. thuringiensis* strains and their biological activity: *B. thuringiensis* strain GM-33 and two well known *B. thuringiensis* strains were inspected under phase-contrast microscopy and proteins from their crystals separated on SDS-PAGE. GM-33 synthesizes a typical bipyramidal crystal (not shown), which is composed of prominent protein band of ca. 130 kDa (Fig. 1A). In order to know the toxicity of crystals from the serovar *monterrey,* we performed bioassays against larvae of three lepidopteran and one Coleopteran species. Mortality results showed that the Mexican strain GM-33, displayed a mortality of 24, 24 and 14% against *H. virescens*, *T. ni* and *S. exigua*, respectively, at dosage of 100 µg. All strains used as positive control gave 100% mortality at both 50 and 100 µg dosage. However HD-511 was negative for both lepidopteran insects.

3.2 Identification of *cry* genes: The *cry* gene content was performed using general primer pairs for the presence of nine *cry* gene classes and *cyt* genes. GM-33 gave a single DNA band at same position that HD-511, which has only *cry*7A gene, as indicated by the production of a 420bp PCR-product with the Un7/8 primers (Fig. 1B). This PCR product was cloned and sequenced. Nucleotide sequence gave a good match, almost 97%, with *cry*7A gene (Data not shown). A new pair of primers were designed according *cry*7A gene sequence, from the promoter to the 420 bp sequenced region (2.2 kbp), and one from this region to the transcriptional terminator (1.5 kbp). The 1.5 kbp and 420 bp fragments were cloned and sequenced (Fig. 2). Deduced amino acid sequence displayed high homology (92%) with *cry*7Aa gene (data not shown). DNA sequence of 2.2 kbp fragment is in progress.

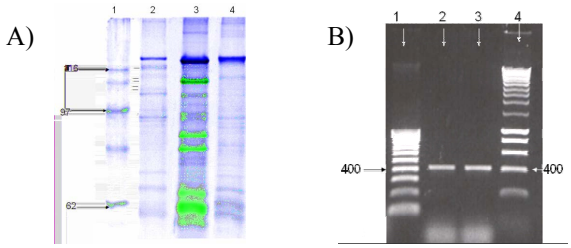

Figure 1: Analysis of the protein profile (A) and the PCR amplicons (B) of the GM-33 and control strains: A) Lanes: 1, molecular weight markers (kDa); 2, HD73; 3, GM-33; 4, HD511. B) Lanes: 1, ladder of 100 bp; 2, HD-511; 3, GM-33; 4, ladder of 200–1000 bp.

3.3 Crystal protein analysis, ICP digestion and N-terminal sequencing: Crystal proteins were separated on polyacrylamide gels and the 130 kDa band was elicited, digested with trypsin and N-terminal protein sequence of three different peptides was performed. Results showed the amino acid sequences 1) TSGYISSGEYSFR; 2) LHGSTDEQDINYNTXXQES; and 3) TPGLVYLL(V)G. Only sequence 1 displayed high homology (92%) with Cry7Aa protein, it mapped in since residue 377. However, other sequences do not show homology with known proteins (data not shown).

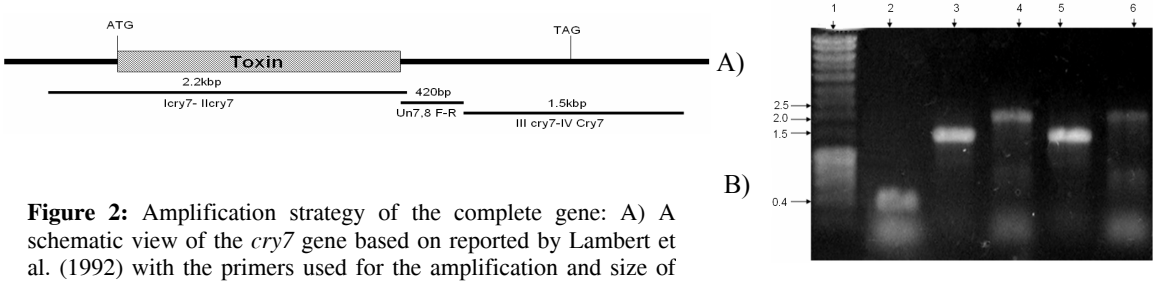

Figure 2: Amplification strategy of the complete gene: A) A schematic view of the *cry7* gene based on reported by Lambert et al. (1992) with the primers used for the amplification and size of the amplicons. B) Lines: 1, DNA molecular size (kbp); 2, HD-511 as figure 1B; 3, HD-511 with primers IIIcry7-IVcry7; 4, HD-511 with primers Icry7-IIcry7; 5, GM-33 with primers IIIcry7-IVcry7; 6, GM33 with primers Icry7-IIcry7.

4. Discussion

In this study, we present the biological activity, identification of putative *cry* gene and characterization of the ICPs from the Mexican *B. thuringiensis* strain GM-33 recognized as holotype for the flagellar serotype H-28a28b serovar *monterrey*. Bioassays showed that GM-33 displayed a marginal effect against *H. virescens* and *T. ni*, but none were toxic against Colorado potato beetle. This strain was negative for β-exotoxin (data not shown), suggesting that the crystal proteins are responsible for the detected biological activity. Prelimnary results with mosquitoes' larvae are consistent with those reported by Guerchicoff *et al* (2002) who found no toxic effects of the GM-33 strain on *Culex* or *Aedes* sp, nor the presence of *cyt* genes encoding proteins with known mosquitocidal activity. PCR results revealed that our strain was negative for *cry*1 genes even that these primers could to detect since *cry*1A to *cry*1K. PCR amplification and their nucleotide sequence, suggest that the GM-33 strain synthesizes a Cry7A-like protoxin. At time we can not explain the reason of the GM-33 toxicity. In early reports were highlighted that proteinases kind, play an important role in correct protoxin activation and this step could to determine the toxicity level against a specific target. Specifically, the Cry7A protein is a good example, because it can be toxic against coleopteran only after it was solubilized and processed with trypsin (Lambert *et al*., 1992). Partial amino acid sequence from three different tryptic fragments of ICP from GM-33, reveled that only labeled as 1 had, almost perfect homology with Cry7A protein at 377 position, but another had any homology with known protein sequence. These results suggest us that GM-33 could harbors a novel *cry* gene, who is be very similar but not identical to *cry*7A gene. Screening based on PCR methodologies represent a power tool to search *cry* genes in a great number of isolates, because it approach give a fast answer on the presence/absence of *cry* genes but PCR does not tell us if genes being present or not in the crystals. For these reasons, bioassays represent the ultimate test to assess the toxicity of an isolated strain. Finally, our results suggest that *B. thuringiensis* strain GM-33 could to synthesize putative novel Cry protein and they ones could display activity against new targets. The characterization of *B. thuringiensis* strains is very important not only to increase the knowledge of this bacterium group but also, because it may help to understand the putative role of *B. thuringiensis* in the environment.

```
1       AAGCAGTGAATGCCTTGTTTACAGAGGGAAGAAATGCACTCCAAAAACACGTGACAGATTATAAAGTGGACCAGGTTTCAATTTTAGTGG
Transl.    A  V  N  A  L  F  T  E  G  R  N  A  L  Q  K  H  V  T  D  Y  K  V  D  Q  V  S  I  L  V

91      ATTGTATATCAGGGGATTTATATCCCAATGAGAAACGCGAACTACAAAATCTAGTCAAATACGCAAAACGTTTGAGCTATTCCCGTAATT
Transl. D  C  I  S  G  D  L  Y  P  N  E  K  R  E  L  Q  N  L  V  K  Y  A  K  R  L  S  Y  S  R  N

181     TACTTCTAGATCCCACATTCGATTGTATTAATTCATCTGAGGAGAATGGTTGGTATGGAAGTAATGGTATTGTGATTGGAAATGGGGATT
Transl. L  L  L  D  P  T  F  D  C  I  N  S  S  E  E  N  G  W  Y  G  S  N  G  I  V  I  G  N  G  D

271     TTGTATTCAAAGGTAACTATTTAATTTTTTCAGGTACCAATGATACACAATATCCAACATATCTCTACCAAAAAATAGATGAATCCAAAC
Transl. F  V  F  K  G  N  Y  L  I  F  S  G  T  N  D  T  Q  Y  P  T  Y  L  Y  Q  K  I  D  E  S  K

361     TCAAAGAATATACACGCTATAAACTGAAAGGTTTTATCGAAAGTAGTCAGGATTTAGAAGCTTATGTGATTCGCTATGATGCAAAACATA
Transl. L  K  E  Y  T  R  Y  K  L  K  G  F  I  E  S  S  Q  D  L  E  A  Y  V  I  R  Y  D  A  K  H

451     GAACATTGGATGTTTCTGATAATCTATTACCAGATATTCTCCCTGAGAATACATGTGGAGAACCAAATCGCTGCGCGGCACAACAATACC
Transl. R  T  L  D  V  S  D  N  L  L  P  D  I  L  P  E  N  T  C  G  E  P  N  R  C  A  A  Q  Q  Y

541     TGGATGAAAATCCAAGTTCAGAATGTAGTTCGATGCAAGATGGAATTTTGTCTGATTCGCATTCATCTTCTCTTAATATAGATACAGGTT
Transl. L  D  E  N  P  S  S  E  C  S  S  M  Q  D  G  I  L  S  D  S  H  S  S  S  L  N  I  D  T  G

631     CTATCAATCACAATGAGAATTTAGGAATTTGGGTGTGGTTTAAAATTCCGACATTAGAAGGATATGCGAAATTTGGAAATCTAGAAGTGG
Transl. S  I  N  H  N  E  N  L  G  I  W  V  W  F  K  I  P  T  L  E  G  Y  A  K  F  G  N  L  E  V

721     ATGAAGATGGCCCAGTTATTGGAGAAGCATTAGCCCGTGTGAAACGCCAAGAAACGAAGTGGAGAAACAAGTTAGCCCAAATGACAACGG
Transl. D  E  D  G  P  V  I  G  E  A  L  A  R  V  K  R  Q  E  T  K  W  R  N  K  L  A  Q  M  T  T

811     AAACCCAGGCGATTTATACACGAGCAAAACAAGCGCTGGAATATCTTTTTGCGAATGCACAAGACTCTCACTTAAAAAGAAATGTTACAT
Transl. E  T  Q  A  I  Y  T  R  A  K  Q  A  L  E  Y  L  F  A  N  A  Q  D  S  H  L  K  R  N  V  T

901     TTGCGGAAATTGCGGCTGCAAGAAAGATTGTCCAATCAATACGCGAAGCGTATATGTCATGGTTATCTGTTGTTCCAGGTGTAAATCACC
Transl. F  A  E  I  A  A  A  R  K  I  V  Q  S  I  R  E  A  Y  M  S  W  L  S  V  V  P  G  V  N  H

991     CTATTTTTACAGAGTTAAGTGGGCGAGTACAACGAGCATTTCAATTATATGATGTACGAAATGTTGTGCGTAATGGTCGATTCCTCAATG
Transl. P  I  F  T  E  L  S  G  R  V  Q  R  A  F  Q  L  Y  D  V  R  N  V  V  R  N  G  R  F  L  N

1081    GCTTATCCGATTGGATTGTAACATCTGACGTAAAGGTACAAGAAGAAAATGGGAATAACGTATTAGTTCTTAACAATTGGGATGCACAAG
Transl. G  L  S  D  W  I  V  T  S  D  V  K  V  Q  E  E  N  G  N  N  V  L  V  L  N  N  W  D  A  Q

1171    TATTACAAAACGTAAAACTCTATCAAGACCGTGGGTATATCTTACATGTAACAGCGCGCAAGATAGGAATTGGGGAAGGATATATAACGA
Transl. V  L  Q  N  V  K  L  Y  Q  D  R  G  Y  I  L  H  V  T  A  R  K  I  G  I  G  E  G  Y  I  T

1261    TTACGGATGAAGAAGGGCATACAGATCAATTGAGATTTACTGCATGTGAAGAGATTGATGCATCTAATGCGTTTATATCCGGTTATATTA
Transl. I  T  D  E  E  G  H  T  D  Q  L  R  F  T  A  C  E  E  I  D  A  S  N  A  F  I  S  G  Y  I

1351    CAAAAGAACTGGAATTCTTCCCAGATACAGAGAAAGTGCATATAGAAATTGGCGGAACCGAAGGGATTTTCCTGGTGGAAGGTATATTAG
Transl. T  K  E  L  E  F  F  P  D  T  E  K  V  H  I  E  I  G  G  T  E  G  I  F  L  V  E  G  I  L

1441    AGGTATTTTTGAGATTGGAGAGCTATGTTATA**TAG**GGAGAGTTTTCCACCAATATTTGTTTGGATTCAAAATAAAATAAAATGCATACAA
Transl. E  V  F  L  R  L  E  S  Y  V  I  ***

1531     TCCTTCTTATTCCAGCGGGTATTCTTAATTAATTATAAATATAGGGTTGGAAAGGTTTAAAAATAAAACCACCGCCTTATTTCCCCATTA
1621     CTAGAAAGTGGAGGGAAGGTACCGTGGTTTTTCCATGAGGTAAAAAAACAAATTAGCGTATATTATCCTTAATTTCTTCCTAATAGAAAG
1711     AAAGCGGGATTTGGATTAAAGAACCCGTAAAGGTGGACAGGGAAATTAGGCATTTTATATCTTATTACCTGCAAAGTCCCAAACAAATGA
1801     GGGTAGTATGAGGTGACAAAAACGCTTTGAAGTTTTCCAAAAAAGAAATCAAGTACAAATTGAAATTAGTACAACAAATGTTATTTCTTT
1891     AGTAGAACGTATAGAATTATTATGTTTGGAAG
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Figure 3: Nucleotide sequence and deduced amino acid sequence of the Cry7-like partial gene (Genebank access: EU274300). The deduced Aminoacid sequence is show in one-letter code.

Acknowledgements: We like to express our gratitude to the Department of Biochemistry and Molecular Biology from the Nebraska Medical School for the protein amino acid sequencing. This work was supported by CONACYT U46730-Z and PAICYT 1541-07. VE. Aguirre-Arzola and A. Alcázar-Pizaña are recipients of CONACYT fellow.

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Identification of actinomycetes with antifungal activity isolated from soil amended with composts

G. Cuesta[*,1], L. Morales[1], R. García de la Fuente[2], S. Botella[1], F. Fornes[2] and M. Abad[2]

[1] Departamento de Biotecnología. Universidad Politécnica de Valencia.46022, Valencia, Spain.
[2] Instituto Agroforestal. Universidad Politécnica de Valencia.46022, Valencia, Spain.

The search for new antifungal biocontrol strategies to inhibit the growth of phytopathogenic fungi has been focused on the genus *Streptomyces* and related species. Actinomycetes were isolated on starch casein agar (SCA), arginine glycerol salts agar (AGSA), and glycerol asparagine agar (GAA), and co-cultured with phytopathogenic fungi in potato dextrose agar (PDA) and yeast extract malt extract agar (YMA). In this work, we have isolated 52 actinomycete strains, 13 of them showing high inhibitory activity against the phytopathogenic fungi tested. Isolated strains were identified by chemotaxonomic procedures, 16S rDNA sequences analysis, and morphological methods. These strains belongs to the species *Streptomyces variegatus, S. griseoruber, S. lusitanus, S. roseogriseus, S. coeruleorubidus, S, griseoruber, S. lincolensis, S. aureoverticilatus*, *S. speibonae*, and *Lechevalieria xinjiangensis.*

Keywords *Streptomyces*, phytophatogenic fungi, composted two-phase olive mill waste

1. Introduction

Actinomycetes are Gram positive bacteria with high GC content in their genome. They are an integral part of the indigenous soil microbiota involved in the turnover of recalcitrant plant organic matter. They are well-known major sources of potentially important secondary metabolites. The search for new antifungal biocontrol systems to inhibit the growth of phytopathogenic fungi has been focused on the genus Streptomyces and related species. About 60% of the products with antifungal activity developed for agricultural use have been isolated from Streptomyces spp. It has been recognized that the control of fungal plant diseases with compost can be as effective as that obtained with chemical fungicides [1]. Different mechanisms have been proposed to explain the control of the plant diseases by compost applications such as competition for nutrients or antibiotic production by beneficial microorganisms [2]. In our search program for actinomycetes producing antifungal antibiotics useful for the control of plant diseases, we have isolated actinomycetes with antifungal activity against phytopathogenic fungi from compost and soil amended with this compost.

2. Material and methods

2.1 Sample collection

Actinomycetes were isolated from one soil sample amended with two composts (based on two-phase olive mill waste mixed with other organic residues) and 1 soil sample without compost. Soil sample were located at 39º45´04´´ in latitude and at 0º41´10´´ longitude.

2.2 Actinomycete isolation

Soils samples and soils amended with compost were homogenized in buffered peptone water at 100 rpm for 30 min. Ten-fold dilutions were made and plated onto starch casein agar (SCA), arginine glycerol salts agar (AGSA), and glycerol asparagine agar (ISP-5) supplemented with cyclohexamide (50 mg/L)[3]. Plates were incubated at 28ºC for 14 and 21 days until sporulated actinomycetes colonies were visible. Isolates were purified in yeast extract malt extract agar (YEME) and stored at 4ºC in slant agar and in 20% glycerol at -20ºC.

2.3 Fungal cultures

Fusarium oxysporum f. sp. *melonis* (CECT 20474), *Phytophthora cinnamomi* (CECT 20186), *Pythium debaryanum* (CECT 2362), *Sclerotinia sclerotiorum* (CECT 2823), *Thanatephorus cucumeris* (CECT 2813) were cultured on potato dextrose agar (PDA) for 5-7 days at 28ºC.

2.4 Microbial antagonism plate assays

Each actinomycete isolated was streaked onto yeast-malt extract agar (YMA) [4] and potato dextrose agar (PDA) [5][6] and incubated at 28ºC for 7 days or until sporulation was observed. Four agar plugs of 0.6 cm in diameter corresponding at four actinomycetes isolated with actively growing were placed onto YMA and PDA medium and incubated for 7 days at 28ºC. Following this time, a plug with actively growing fungal mycelia was placed in the centre of the plate and cultured at 28ºC for 7-14 days. Antagonism was determined as the distance between actinomycete growth and fungal growth. The distance between the actinomycetes plug and fungi plug was 3 cm.

2.5 Identification of antagonistic actinomycetes

Actinomycete strains were growth on YEME medium for 5 days at 28ºC. The isomers of diaminopimelic acids, the sugar composition of whole-cell walls, mycolic acids were determined by standardized procedures [7][8][9]. Total genomic DNA from pure culture colonies was extracted following the CTAB procedure and subjected to PCR amplification with primers 27f and 1525r [10]. Reactions were performed in a final volume of 25 µl containing 0.2 mM of each of the four dNTPs (Ecogen), 0.4 µM of primer 27f and primer 1525r, 1 µl extracted DNA, 1.5 µM $MgCl_2$, and 1.25 U *Taq* DNA Polymerase (Ecogen) with 1× reaction buffer (10× buffer: 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25ºC), 0.1 % Tween-20). Amplification was performed in a PTC-100 Peltier Thermal Cycler as follows: after an initial denaturation step (95ºC, 5min), 30 cycles of denaturation (95ºC, 1 min), annealing (54ºC, 1min), and extension (72ºC, 1 min) were performed, followed by a final extension (72ºC, 10 min). Controls without template DNA and without DNA were included in each PCR experiment. The PCR product was purified with the Gen Elute PCR Clean-up Kit (Sigma) and sequenced with the same sets of primers using an ABI PRISM® BigDye™ Terminator Cycle sequencing kit (version 3.1). Sequencing gel electrophoresis was carried out and the nuceotide sequences were automatically obtained by using an Applied Biosystems 3730xl DNA Analyzer. The 16S rRNA gene sequences were manually assembled from the combination of separate fragments generated with forward and reverse sequencing primers using the PHYDIT program [11] and were obtained an almost complete sequence of each 16S rRNA gene. With these sequences actinomycete identification was carried out using the program BLAST (Basic Local Alignment Tool). Aerial spore-mass colour, substrate mycelial pigmentation and the production of diffusible pigments were recorded on the International *Streptomyces* Project culture media (and incubated 28ºC for 14 days). The spore-chains morphology was examined by light microscopy of 10-14 days cultures growth on glycerol asparagine agar (ISP-5). For the detection of chitinase activity, actinomycetes strains were streaked on agar plate medium containing 0.2% colloidal chintin, 0.05% KCl, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, 0.05% yeast extract, and 2.0% agar (pH 7.0) and were incubated at 28ºC for 3 to 14 days. Chitinase production was assessed by visual inspection of cleared zones formed around colonies [12].

3. Results and discussion

We have isolated 52 actinomycete strains using the selective media SCA, AGSA and ISP-5, thirteen of them showing a high inhibitory activity against the phytopathogenic fungi tested. In Table 1 is shown the antagonistic pattern of this isolates. Mayor inhibition (+++) was scored as a separation between actinomycetes and fungi of 2 cm, intermediate inhibition (++) as a 1 cm, and a minor inhibition (+) was scored as 0.5 cm separation. No inhibition was scored when contact between actinomycetes and fungi existed.

All isolates, except strain T2-19, were presumptively assigned to the genus *Streptomyces* by their characteristic colonies and pigmentation properties. All the actinomycete strains produced moderate to abundant aerial hyphae, however strain T2-19 produced scant aerial hyphae. They are aerobic, Gram-positive, and fragmentation of mycelium did not occur. Colours of the isolated strains were ranging from orange, brown, to yellow. Amino acids in the peptidoglycan layer in all strains were LL-diaminopimelic acid. Mycolic acids were not detected. Melanin pigments were produced in 8 of the strains.

Table 1 Antifungal activities of the actinomycetes isolated that showed the highest antagonistic pattern in YMA and PDA media

Actinomycete strain	*Fusarium oxysporum* f. sp. *melonis*		*Phytophthora cinnamomi*		*Sclerotinia sclerotiorum*		*Pythium debaryanum*		*Thanatephorus cucumeris*	
	YMA	PDA	YMA	PDA	YMA	PDA	YMA	PDA	YMA	PDA
CO2-9	-	-	++	+	-	-	-	-	++	+++
CO2-16	++	++	+++	+++	+	+++	+++	+++	+++	+++
ME-5	-	++	-	++	-	-	-	+++	-	+
S-1	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
S-2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
S-3	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
S-5	++	++	++	++	+	++	+	++	+++	+++
S-6	+++	+++	+++	+++	+++	+++	+	+++	+++	+++
S-7	-	+++	-	+++	-	+++	+++	+++	+++	+++
T2-10	-	-	+++	+++	-	-	-	-	++	++
T2-19	-	-	++	++	+	-	+	+	+	+
T6-32	-	-	++	-	++	-	++	-	+	-
T8-2	-	+	-	+	-	+	-	+	++	++

Table 2 shows the identification resulting from 16S rRNA gene sequence analysis and phenotypic test. As a result, the 13 isolated strains with inhibitory activity belonged to the species *Streptomyces variegatus, S. griseoruber, S. lusitanus, S. roseogriseus, S. coeruleorubidus,, S. lincolensis, S. aureoverticilatus*, *S. speibonae* and *Lechevalieria xinjiangensis*. Six of these species (CO2-16, S-1, S-2, S-3, S-5, S-6, and S-7) showed the highest antagonistic activity against the five phytopathogenic fungi. In these cases the maxim activity was detected in PDA medium. It is remarkable that *L. xinjiangensis* T2-19, although not shows the maxim activity, is able to inhibit the growth of four from the five fungi. *Lechevalieria* was proposed in 2001 [13] and, at present, it is not known the antagonistic activity against fungi. This strain showed chitinase activity and this could be the mechanism for fungi inhibition. Chitinolitic activity is well known in higher plants and *Streptomyces,* but there aren't any paper describing the chitinolitic activity in *Lechevalieria*; therefore, this genus can be a new agent of control of phytopathogenic fungi. In the last decade researches have been focussed on rare genera of actinomycetes as producers of new activities. In this study we showed a known activity in *Streptomyces* such a chitinase in a relatively new genus such as *Lechevalieria.*

Table 2 Identification of actinomycete strains by 16S rDNA analysis and phenotypic test

Identification by analysis of 16S rDNA sequences	DAP isomer	Aerial spore mass colour	Melanin production	Difusible pigment	Chitinase production
S. lincolnensis CO2-9	L	Light green	+	+	-
S. aureoverticillatus CO2-16	L	Dark orange	+	-	-
S. speibonae ME-5	L	Light grey	-	-	-
S. variegates S-1	L	Dark orange	+	-	-
S. variegates S-2	L	Orange-red	+	-	-
S. variegates S-3	L	Light orange	+	-	-
S. griseoruber S-5	L	Blue-green	+	+	-
S. lusitanus S-6	L	Dark yellow	-	-	-
S. roseogriseus S-7	L	Light grey	-	-	-
S. coeruleorubidus T2-10	L	White green	+	+	-
L. xinjiangensis T2-19	Meso	Beige	-	-	+
S. griseoruber T6-32	L	Blue-green	-	+	-
S. griseoruber T8-2	L	Blue-green	+	-	-

4. Conclusion

It has been widely demonstrated that actinomycetes play an important role in the last phase of the composting process due to its ability for the degradation of recalcitrant organic compounds. The use of actinomycetes with

antifungal activity like inoculum in composting can offer new possibilities in the control of phytopatogenic fungi. Although there are a lot of reports about the suppressive effects of compost, no information is currently available about the use of antagonistic actinomycetes like a starter culture for composting; therefore, further studies should be made in order to investigate the role of antifungal actinomycetes in the last phase of composting and its activity "in vivo".

Acknowledgements This research was carried out within the framework of projects PTR1995-0894-OP-02-02 and UPV-PPI-05-05-5542 subsidized by Spanish Ministry of Education and Science (MEC) and the Polytechnic University of Valencia (UPV), respectively.

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Identification of *Mycobacterium* sp. as Alfalfa endophytes using 16s rRNA gene sequence analysis

A. Deljou[1], K. Mousavi[1], A. Ghasemi[2]and H. Rahimian[3]

[1] Faculty of Agriculture, BuAli-Sina University,Hamedan, Iran
[2] Evin Plants Pests & Diseases Resrch Center , Tehran Iran
[3] Faculty of Agriculture, Mazandaran University, Sari, Iran

In a study performed to assess Alfalfa bacterial vascular diseases, we isolated bacterial endophytes as associated agents with bacterial vascular disease, residing in root tissues and identified them as Mycobacterium sp. based on 16s rRNA sequence analysis and protein SDS-PAGE profiling. Comparison of 16s rRNA sequences with those previously deposited in databases, showed >%99 sequence similarity with Mycobacterium species. Mycobacterium sp. have been previously isolated from some agronomic crops and perennial plants like wheat and scot pine, but this may be the first report of isolation of Mycoabcterium sp. as endophytes from alfalfa root.

Keywords Mycobacterium sp; 16s rRNA

Introduction

Various kinds of microorganisms have been found inside plants. These microbes (Endophytes) include fungi, actinomycetes and bacteria (1). Microbial endophytes are defined as microorganisms detected inside surface sterilized plants (2). Endophytes can be either pathogenic or non-pathogenic to their host (1).

Both gram positive and gram negative bacterial endophytes have been isolated from several tissue types in numerous plant species (3). Furthermore, several different bacterial species have been isolated from a single plant (3). The microorganisms can reside within cells (4), in the intercellular spaces (5) or in the vascular system (6).

Mycobateria are gram positive actinobacteria that are distributed widely in water and soil (7) and reported to be one of the 14 most abundant genera from soils, accounting for about 2.6% of total soil habitants (8).
In previous studies mycobacterium sp. had been isolated as endophytes from Wheat (9) and Scots pine (10). In this study, as a result of surveying alfalfa wilt disease, we found Mycobacterium sp. as endophytic microorganisms in alfalfa roots.

Materials and Methods

Isolation of Endophytic Bacteria
Plant samples were collected from different Alfalfa fields of Hamadan province (Iran). Samples were transferred to the laboratory and were thoroughly washed with running tap water to remove soil particles. Roots were cut and surface sterilized with ethanol 70% for 1.5 minutes, Sodium Hypochlorite %3.75 for 6 m and then ethanol 70% for 45s. Root segments were rinsed three times with sterile distilled water to remove completely hypochlorite from root surface. Roots were chopped into 0.5 cm pieces and were suspended in 2 ml sterilled double distilled water. Resulted suspension were streaked on Corynebacterium agar and placed in incubator with temperature 22-24 C. Appearing creamy yellow colonies after 7-8 days were picked and were smeared on Corynebacterium agar medium and purified.

Biochemical Tests

Oxidase reaction was done according to Kovacs. Catalase reaction, gelatin, esculin, starch, casein of milk and tween80 hydrolysis, levan production, DNAase test and motility were done according (11).

DNA Extraction

Isolates were cultured on liquid medium and were shaked at 26-27C° for 3 days. DNA was extracted by the method of (12).

PCR Amplification and 16s rRNA Gene Sequencing

The eubacterial universal primers fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC) (13) were used for amplification of 16s rRNA gene from bacterial isolates. Each reaction mixture contained 1X PCR buffer, 2.5 unit Taq DNA polymerase, 1.5 mM magnesium chloride, each dNTPs at a concentration of 0.2 mM, each primer at a concentration of 0.1 mM and 10 ng DNA in a 50µl PCR tube The thermocycling reactions consisted of a denaturation step at 94 C˚ for 3 min, 30 amplification cycles of 93 C˚ for 1 min, 53 C˚ for 1 min, and 70 C˚ for 1 min and a final polymerization step of 70 C˚ for 7 min.with an Eppendorf PCR system. Sequencing was performed using fD1 primer by an automated sequencer (13).

Phylogenetic Analysis

Approximately 850 bp was used for phylogenetic analysis. The phylogenetic position of the isolates were determined by searching NCBI databases with the BLAST program. Multiple alignment was done by ClustalX program and the phylogenetic tree was made by MEGA4 software using neighbour-joining method with 2000 bootstrap replicates.

Results and Discussion

Biochemical Tests

Catalase, oxidase, citrate and tween80 hydrolysis tests were positive. Other performed tests were negative and some of them were variable. Results of biochemical tests are shown in Table 1.

PCR reaction resulted in a single 1.5 kb band and this indicates that the universal primers, specifically amplified the entire 16s rRNA gene with a size of 1.5 kb (figure 1). Nearly 850 bp of 16s rRNA gene sequence was determined with fD1 primer and was used in phylogenetic analysis.

Sequences were searched in NCBI databases by BLAST program and the sequences most similar to the isolates were found. The partial 16s Rrna gene sequence of the isolates showed the most similarity with Mycobacterium sp., strain EF405863.1. The 16s Rrna gene sequence of the samples were 99-100% similar to those of Mycobacterium sp. Based on 16s Rrna sequence data and the phylogenetic tree made of these sequences, the isolates formed a monophyletic cluster with Mycobacterium sp. strain EF405863.1., paraphyletic to those of other Mycobacterium sp. The most variable regions of 16s r RNA gene in Mycobacterium sp. are the regions between bases 180-420 (14) that sequencing of these regions could be used for isolate identification and in most papers, >99% similarity is assumed as intra-species value (15). Based on blast search of isolates sequences and the resulted phylogenetic tree, the isolates belong to Mycobacterium sp (figure 2).

The habitat inside plants is a dynamic environment in which many factors affect structure and combination of microbial species (16). The endophytic populations of a variety of plants are affected by a number of biological and environmental factors such as plant cultivar, plant age, tissue type, time of sampling, and soil environment (9). In many articles, there are reports on endophytic nature of human bacterial pathogens and on the contrary, human tissues infection by bacterial plant pathogens. In a study funke et al. (2005) found curtobacterium sp. Especially C. flaccumfaciens from human clinical specimens (17). Some strains of Curtobacterium flaccumfaciens are endophytic plant pathogens. In other cases, endophytes have been found to be closely related to human pathogens or are either human or opportunistic human pathogens. This is the case of endophytic Salmonella strains, which have caused outbreaks and constitute a health risk for consumers of raw fruits and vegetables (18), and of the Burkholderia cepacia strains isolated from plants (19). As Burkholderia cepacia causes pulmonary infection (even fatal) in human cystic fibrosis patients, a reassessment of the risk and a moratorium on the agricultural use of Burkholderia strains have been suggested. Nocardia endophytes have been isolated from members of genus Citrus. Some Nocardia species are known to be human pathogens causing nocardiasis that is transmitted by soil. In previous studies mycobacterium sp. have been isolated from Wheat and Scots pine, but this may be the first report of identifying Mycobacteria in Alfalfa (Medicago sativa). Since, some of Mycobacterium sp. are important and dangerous human and animal pathogenic factors, discovering these species in alfalfa roots as endophytes could be of great importance for human health and nutrition. Following these results and results of previous studies, it is recommended to better recognize crop plants endophytic bacterial populations, because some of them could be of great importance for human and livestock health.

Table 1- Biochemical tests of bacterial isolates

	Isolates											
Test	**Feiz1**	**Feiz2**	**Amz2**	**Jahan**	**Zamar**	**Var2**	**Koz2**	**Mosle**	**Gh1**	**Gh2**	**Zamar**	**Gh3**
Pathogenicity	+	+	+	+	+	+	+	+	+	+	+	+
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Colony color	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**	**CY**
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-
Catalse	+	+	+	+	+	+	+	+	+	+	+	+
Oxidative	+	+	+	+	+	+	+	+	+	+	+	+
Movement	-	-	-	-	-	-	-	-	-	-	-	-
citrate	+	+	+	+	+	+	+	+	+	+	+	+
Glatin	-	-	-	-	-	-	-	-	-	-	-	-
Casein	-	-	-	-	-	-	-	-	-	-	-	-
Esculin	**W**	**W**	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-
Twee- 80	+	+	+	+	+	+	+	+	+	+	+	+
DNAase	-	-	-	-	-	-	-	-	-	-	-	-
Levan	-	-	-	-	-	-	-	-	-	-	-	-
Asetate	-	-	-	-	-	-	-	-	-	-	-	-
Format	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
Riboze	-	-	-	-	-	-	-	-	-	-	-	-

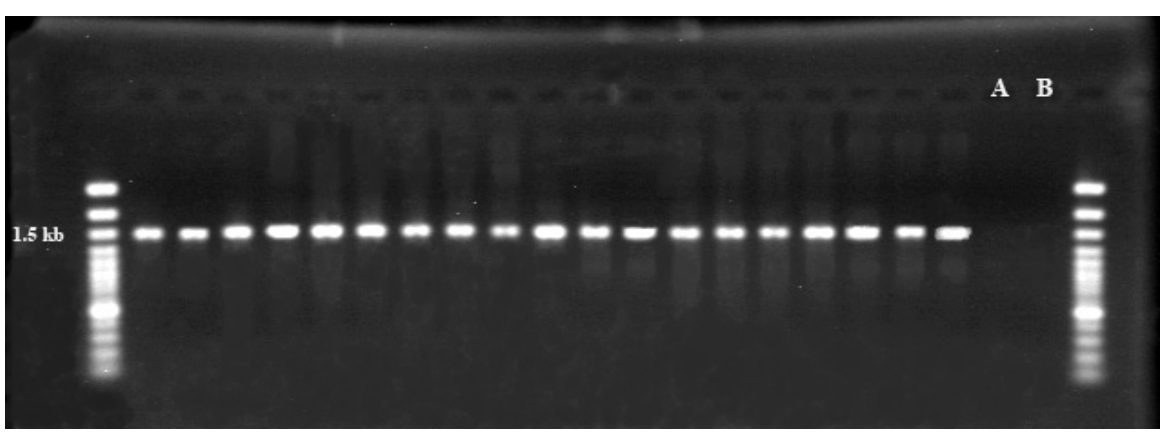

Figure 1: Gel electrophoresis of PCR products

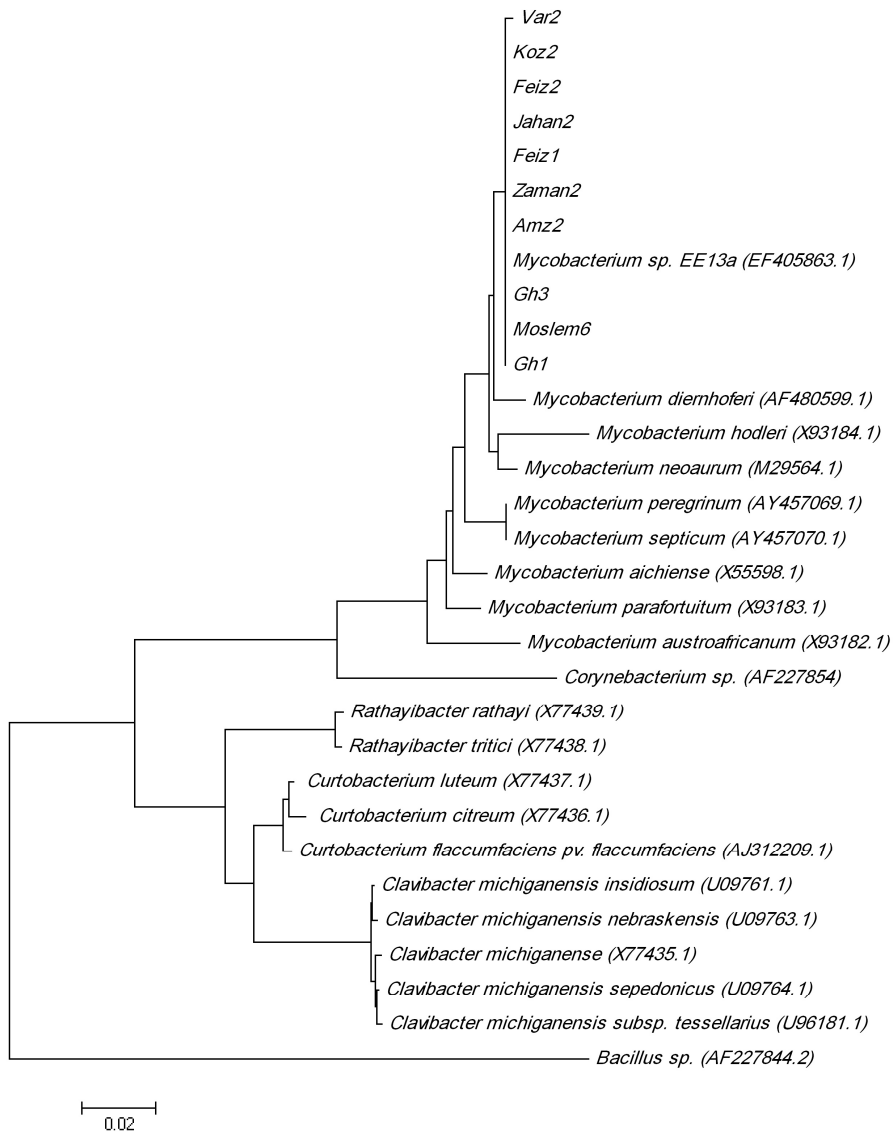

Figure 2: Phyologenetic tree based on 16s rRNA GENE

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Identification of the respiratory chain of *Armillaria mellea* (A.m.) in mushroom state and cultured *in vitro*

L. Feize[1], D. Minai-Tehrani[2], B. Behboudi[1], and **E. Keyhani[3,*]**

[1] Biology Department, Faculty of Sciences, University of Tehran, Iran
[2] Biology Department, Faculty of Sciences, Shahid Beheshti University, Tehran, Iran
[3] Laboratory for Life Sciences, Saadat Abade, Sarve Sharghi 58, 19979 Tehran, Iran

[*]Corresponding author: e-mail: keyhanius2002@yahoo.com

A.m. is a world-wide pathogen for conifers, ornamental trees as well as fruit trees such as *Prunica persica*. Cytochromes *c* oxidase, *c* and *b* were detectable in mitochondria isolated from A.m. rhizomorphe grown in semi-solid malt-agar-sucrose medium. The mitochondria oxidized NADH, succinate and TMPD-ascorbate; rotenone, antimycin A, and KCN inhibited NADH, succinate and TMPD-ascorbate oxidation, respectively. In mitochondria isolated from rhizomorphe grown in liquid medium with glucose, cytochrome *c* oxidase was not detectable, while cytochromes *b* and *c* were present. In mitochondria isolated from A.m. rhizomorphe grown in liquid medium with ethanol, or from A.m. mushroom (carpophore, cap and stem), all three cytochromes were present. Data showed that A.m. in the mushroom state or cultivated in vitro exhibited all cytochromes, but that it lacked cytochrome *c* oxidase when cultivated with glucose, suggesting an alternative electron transport pathway in its mitochondria.

Keywords respiratory chain; mitochondria; mushroom; rhizomorphe; *Armillaria mellea*

1. Introduction

Armillaria mellea (Fr. Ex Vahl) Quelet (A.m.) is a world-wide pathogen of broad leaved trees, conifers and numerous other woody ornamental trees, including fruit species. It thrives as parasite on living host tissue or as saprophyte on dead woody material [1]. It has caused devastation and extinction of many oak trees forests and extensive tree mortality in peach [2, 3]. The roots are A.m.'s common habitat, rendering its detection difficult unless characteristic mushrooms are produced around the base of the tree or symptoms such as thin foliage, discoloration, are seen in the branches [3]. In vitro culture has been done in various media [4, 5]. Furthermore, methods for the identification of various Armillaria using isozymes have been worked out [6].

The purpose of the present investigation was to study the respiratory chain of A.m. in the mushroom state, from its natural habitat, as well as in the rhizomorphe state cultured under various experimental conditions. We were able to isolate for the first time mitochondria from A.m. in the mushroom state or from A.m. rhizomorphe cultured in vitro, and we showed that they contained all the cytochromes present in the mitochondria of plants and animals, except for rhizomorphes grown in liquid medium with glucose. Mitochondria from A.m. rhizomorphes cultured with glucose as substrate lacked cytochrome *c* oxidase, suggesting that there was an alternative pathway of electron transport in these mitochondria.

2. Materials and Methods

Clusters of A.m. mushroom were harvested from the Botanical Garden of the Agricultural Institute of Karaj, Iran. In vitro cultures were done according to reference [7], fully described by Feize [8]. Briefly, culture was done 1) in Petri dishes on semi solid agar-malt sucrose medium; 2) in tubes, in liquid culture media containing various salts, peptone and either 2% glucose as substrate, or ethanol as substrate (20 ml ethanol in 100 ml medium). All cultures were done at 25°C and in the obscurity, for 20 days.

Mitochondria were isolated in a buffer that was 0.22 M mannitol, 0.2 M sucrose, 0.2 mM EDTA, 0.4 mM morpholinopropane sulfonic acid (MOPS), with 0.2 mg/ml phenylmethanesulfonyl fluoride as protease inhibitor. A.m. mushroom (carpophore, cap or stem) or rhizomorphe was homogenized in a Waring blender and the homogenate centrifuged 10 min at 700 g to remove nuclei and unbroken cells. The supernatant was centrifuged 10 min at 1,400 g to collect heavy mitochondria, and re-centrifuged 30 min at 8,000 g to collect light mitochondria.

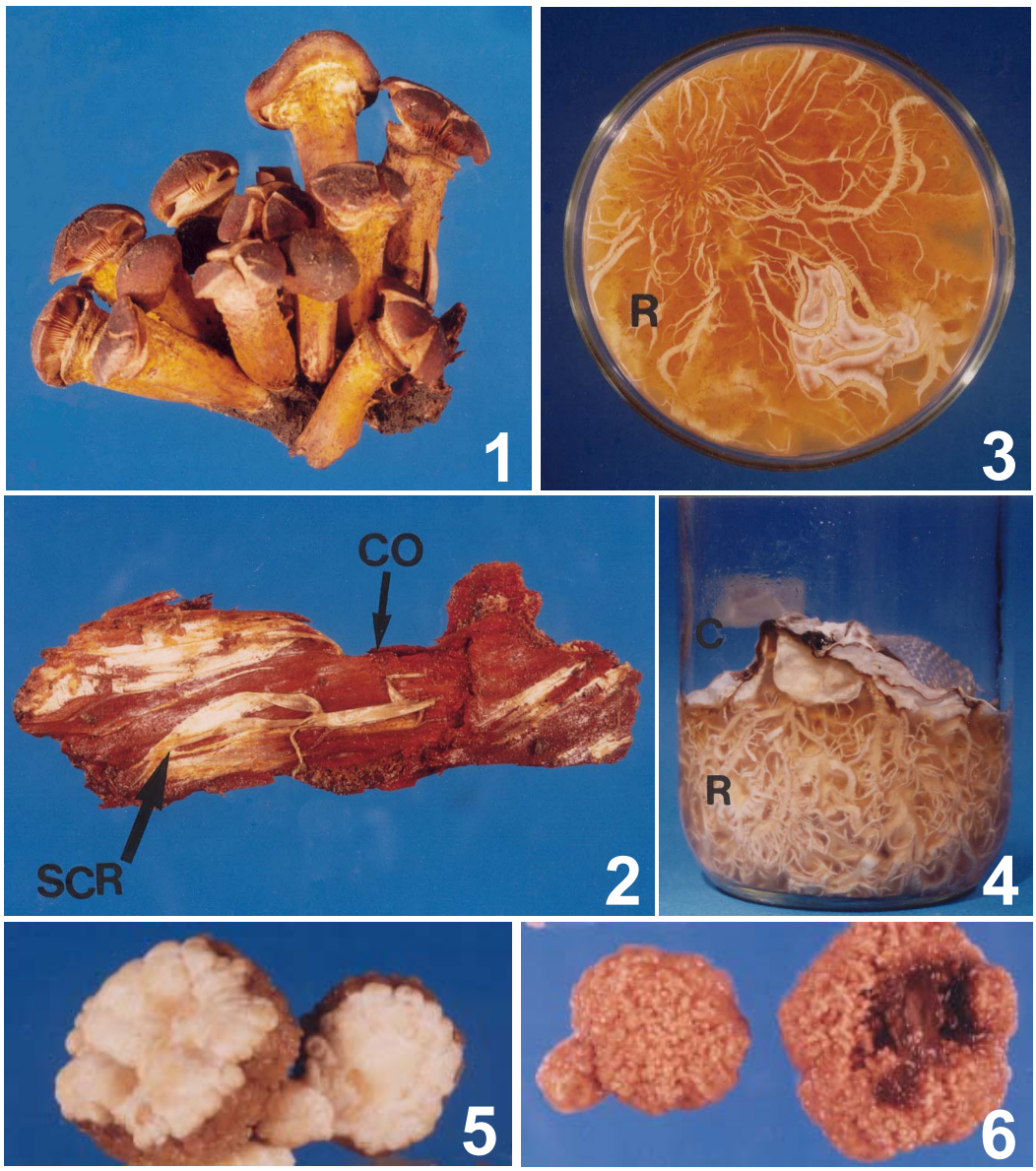

Fig. 1 Cluster of A.m. mushroom from natural habitat. **Fig. 2** A.m. found on a decaying wood (CO); SCR : subcutaneous rhizomorphe. **Fig. 3** A.m. rhizomorphe (R) grown in semi-solid agar-malt-sucrose medium; note the formation of a network of rhizomorphe. **Fig. 4** A.m. grown in liquid peptone-glucose medium; note the extensive rhizomorphe (R) ramification. **Figs. 5 and 6** The two faces of A.m. cultivated in liquid medium containing ethanol as substrate: exterior surface (Fig. 5) and interior surface that is immersed in the medium (Fig. 6). Note that the rhizomorphe is compact in this case. For details, see text.

Combined heavy and light mitochondria were washed once in homogenization medium and centrifuged 30 min at 12,000 g. The pellet was used for spectrophotometric studies. Mitochondria isolation from the mushroom parts was relatively easy, while rhizomorphe mitochondria isolation was extremely difficult and tedious.

Spectrophotometric studies (reduced-minus-oxidized and pyridine hemochromogen difference spectra of mitochondria suspensions) were done exactly as described in reference [9].

3. Results

3.1 Morphology in natural habitat and in vitro

Figure 1 shows a cluster of A.m. mushrooms having a definite cap and, just below the cap, an annulus membrane followed by the stem. Figure 2 shows A.m. on the surface of decaying wood. Figure 3 shows A.m. rhizomorphe cultured in semi solid agar rich medium. The rhizomorphe radiated from a central region. In liquid medium, abundant formation of rhizomorphes was seen when glucose was the substrate (Fig. 4), but small and compact rhizomorphes were seen when ethanol was the substrate (Figs 5 and 6). Figure 5 shows the air-exposed A.m. face; figure 6 shows the medium-exposed A.m. face.

3.2 Respiratory chain

Figure 7A shows the reduced-minus-oxidized difference spectrum of mitochondria from A.m. carpophore produced in natural habitat and Figure 7B shows the spectrum of mitochondria from A.m. rhizomorphes grown in semi-solid rich medium. In both cases, the α absorption bands at 605, 560 and 550 nm due, respectively, to cytochromes *c* oxidase, *b*, and *c*, were seen; the absorption band at 445 nm, due to cytochrome *c* oxidase and that at 430 nm due to all cytochromes, were seen as well. Pyridine hemochromogen difference spectrum of mitochondria isolated from the rhizomorphes (Fig. 7C) showed an absorption band at 588 nm due to the heme of cytochrome *c* oxidase and an absorption band at 555 nm due to the hemes of cytochromes *b* and *c*. The spectra of mitochondria isolated from rhizomorphes cultured in various media showed patterns similar to those described above, except for mitochondria from rhizomorphes cultured in liquid medium with glucose as substrate. These mitochondria contained cytochromes *b* and *c*, but lacked cytochrome *c* oxidase as evidenced by their spectrum exhibiting the bands at 560, 550 and 430 nm, but lacking the bands at 605 and 445 nm (Fig. 7D). Similarly, their pyridine hemochromogen spectrum lacked the absorption band due to cytochrome *c* oxidase (Fig. 7E).

The amount of cytochromes in mitochondria isolated from mushroom parts and from rhizomorphes cultured in various media was calculated as nmoles /mg prot. (Fig. 8). The highest amount was in rhizomorphes from liquid medium with ethanol as substrate, followed by rhizomorphes from semi-solid medium. For mushroom parts (from natural habitat), the highest amount was in carpophores, closely followed by caps and, further down, by stems.

Mitochondria from A.m. mushroom or from A.m. rhizomorphes cultured either in semi-solid agar medium or in liquid medium with ethanol as substrate, oxidized 3 mM NADH, 10 mM succinate, and 0.8 μM N,N'-tetramethyl-p-phenylenediamine (TMPD)/70 μM ascorbate. The oxidations were inhibited by, respectively, 1 μM rotenone, 15 μg/ml antimycin A, and 1.5 mM cyanide.

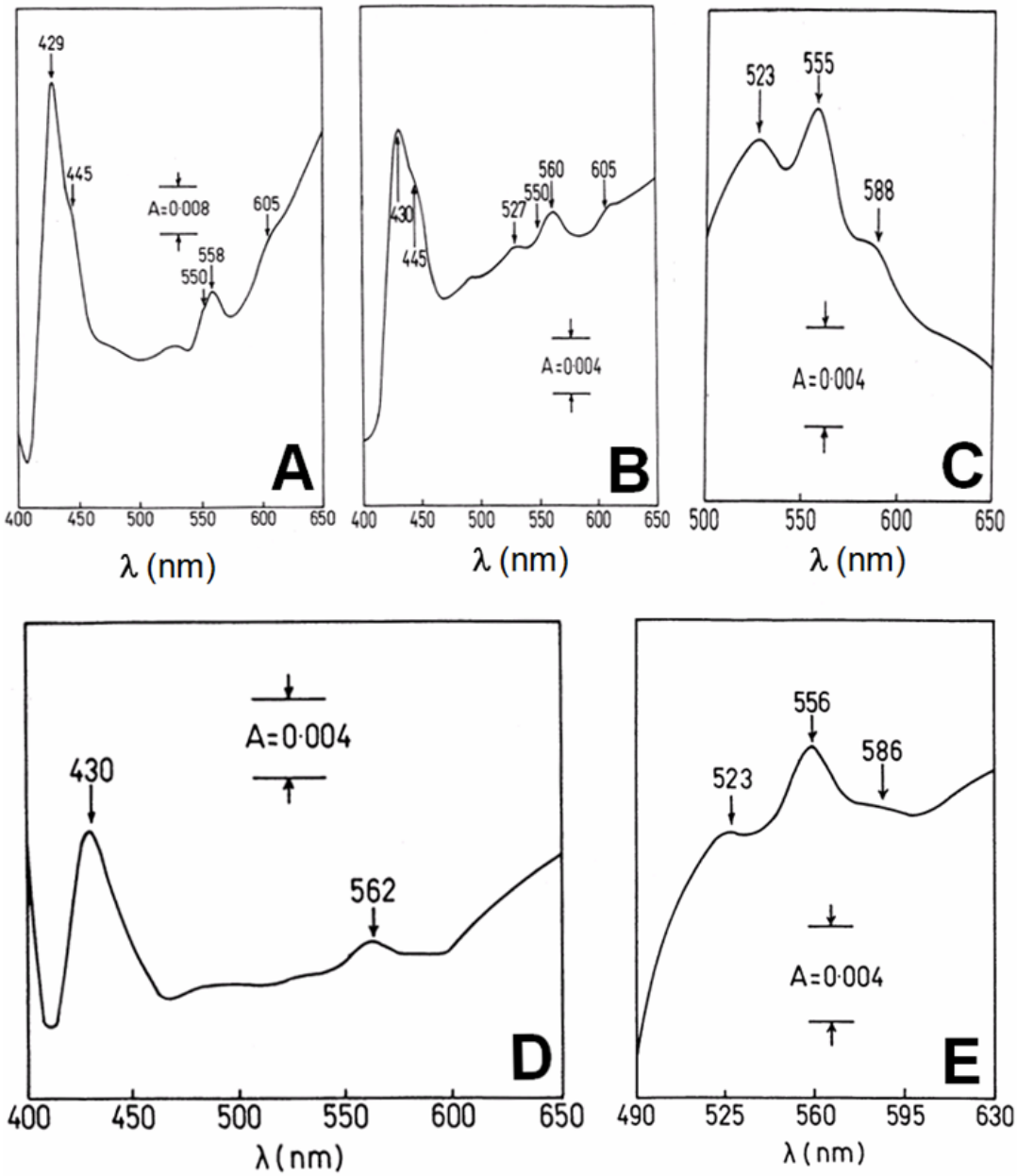

Fig. 7 Reduced-minus-oxidized difference absorption spectra of mitochondria from A.m. mushroom carpophore (A) and from A.m. rhizomorphes cultured in semi-solid agar medium (B); note the presence of cytochromes *c* oxidase at 605 nm, *b* at 560 nm, and *c* at 550 nm. Pyridine hemochromogen difference spectrum of mitochondria from A.m. rhizomorphes cultured in semi-solid agar medium (C); the absorption band at 588 nm is due to cytochrome *c* oxidase and that at 555 nm is due to cytochromes *b* and *c*. Reduced-minus-oxidized difference absorption spectrum (D) and pyridine hemochromogen difference spectrum (E) of mitochondria isolated from A.m. rhizomorphes cultured in liquid medium with glucose as substrate; note the absence of cytochrome *c* oxidase in both spectra.

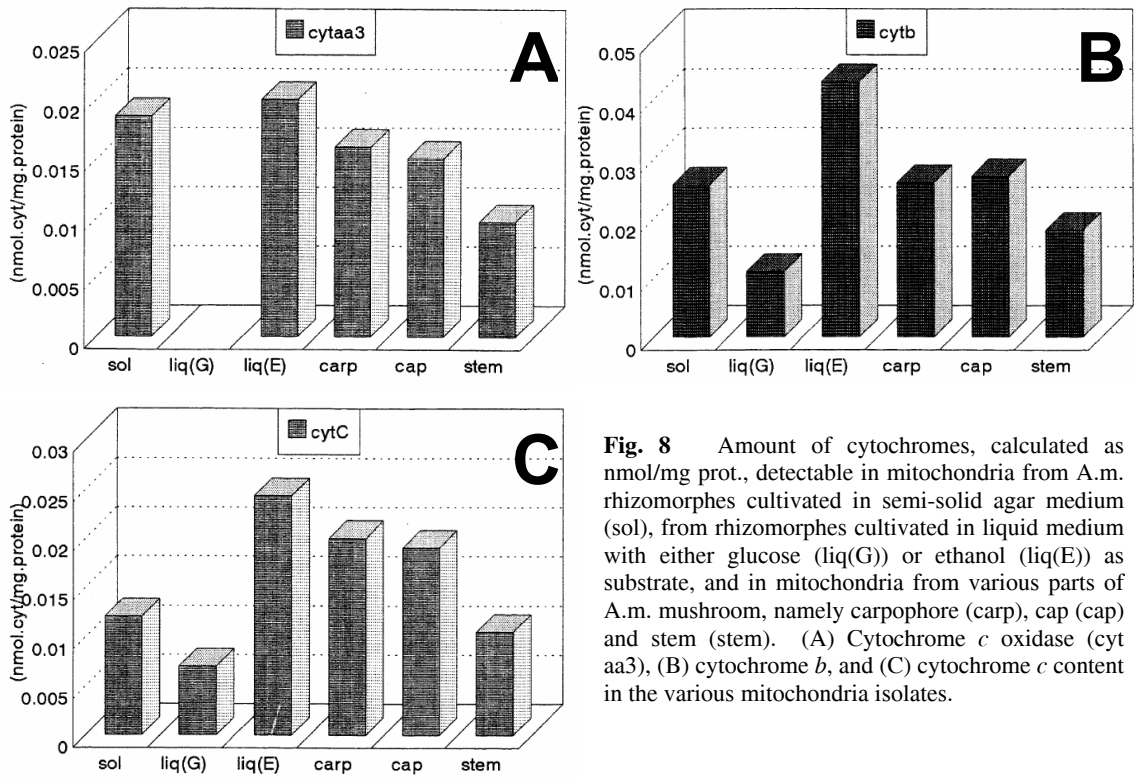

Fig. 8 Amount of cytochromes, calculated as nmol/mg prot., detectable in mitochondria from A.m. rhizomorphes cultivated in semi-solid agar medium (sol), from rhizomorphes cultivated in liquid medium with either glucose (liq(G)) or ethanol (liq(E)) as substrate, and in mitochondria from various parts of A.m. mushroom, namely carpophore (carp), cap (cap) and stem (stem). (A) Cytochrome *c* oxidase (cyt aa3), (B) cytochrome *b*, and (C) cytochrome *c* content in the various mitochondria isolates.

4. Discussion

Our results showed the polymorphism of A.m. cultured in vitro. Depending on the substrate, it either showed a large mass of rhizomorphes extending as a function of culture surface availability, or a compact mass of tissue with small rhizomorphes. Furthermore, depending on the culture medium and substrate used, A.m. showed different respiratory chains. A.m. mushroom collected in the field had the same set of cytochromes than A.m. cultured in semi-solid medium or in liquid medium with ethanol as substrate. Moreover, substrate oxidation and sensitivity to inhibitors exhibited by these mitochondria suggested that the electrons cross-over in the respiratory chain was similar to that found in mitochondria of other organisms. An interesting result was that cytochrome *c* oxidase was undetectable in A.m. cultured in liquid medium with glucose as substrate. This suggested the existence of an alternate electron transport chain and terminal oxidase, presumably insensitive to KCN, as has been demonstrated for other organisms [10, 11, 12]. As seen for A.m. grown in liquid medium with glucose as substrate, sugars have been shown to regulate the concentration and activity of alternate oxidase in *Poa annua* roots [13].

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Isolation and identification of sulphur-oxidizing bacteria from composted two-phase olive mill waste amended with elemental sulphur

R. García de la Fuente[1], **E. Sanchis-Jiménez**[2], **F. Fornes**[1], **S. Botella**[2], **G. Cuesta**[2], and **M. Abad**[*,1]

[1] Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, 46022 Valencia, Spain
[2] Departamento de Biotecnología, Universidad Politécnica de Valencia, 46022 Valencia, Spain

[*]Corresponding author: e-mail: mabad@prv.upv.es, Phone: +34 963877336

A strongly alkaline compost prepared with two-phase olive mill waste ("alperujo") was amended with elemental sulphur (S^0) to assess the contribution of autotrophic bacteria, heterotrophic bacteria, actinomycetes and fungi to the acidification process, and to allow the isolation and identification of sulphur-oxidizing bacteria. S^0 application decreased pH by 3 units and increased remarkably the growth of autotrophic bacteria, whereas it did not affect significantly heterotrophic bacteria, actinomycetes and fungi populations. Five potential sulphur-oxidizing strains were isolated from the amended compost and identified as sulphur-oxidizing bacteria: 3 strains as *Paracoccus thiocyanatus*, 1 as *Halothiobacillus neapolitanus*, and 1 as *Pseudomonas stutzeri*. Finally, an inoculation experiment with sterilized compost revealed that compost native sulphur-oxidizing bacteria showed higher acidification efficiency than reference strains which were not present in the original compost.

Keywords compost pH; acidification; tiobacilli; alperujo

1. Introduction

The olive oil extraction industry has a considerable economic importance in most Mediterranean countries, Spain being the main olive oil producer with a mean production of 1.1 million tonnes per year in the period 2002 to 2006.

During the last two decades the oil extraction technology has evolved from the three-phase to the two-phase centrifugation system, thus reducing substantially the production of highly pollutant wastewater and providing a more efficient use of natural resources. The introduction of the two-phase decanter –adopted by 90% of the Spanish olive mills– entails the production of a new by-product, the two-phase olive mill waste (TPOMW, also called "alperujo"). TPOMW is an organic solid material, with low porosity, sludgy texture and high humidity, which also contains fats and phenols. This by-product cannot be used directly as an organic amendment due to nitrogen immobilization risks and its phytotoxic effects [1]. Nevertheless, TPOMW can be appropriately composted for its use in agriculture [2]. The resulting compost is strongly alkaline (achieving pH values greater than 8.5) for its application as soil improver and hence acid amendments, such as elemental sulphur (S0), are required to reduce the pH for horticultural purposes. During the acidification process powdered S0 is oxidized to sulphuric acid by different microorganisms, especially sulphur-oxidizing chemoautotrophic bacteria. In addition, chemoheterotrophic microorganisms, such as fungi, actinomycetes and bacteria may also contribute to sulphur oxidation [3].

The aim of this investigation was to evaluate the contribution of autotrophic bacteria, heterotrophic bacteria, actinomycetes and fungi to the acidification process of composted two-phase olive mill waste when amended with S0, and to isolate and identificate sulphur-oxidizing bacteria from this amended compost.

2. Materials and methods

2.1 Compost preparation and characterization

TPOMW compost was prepared by co-composting two-phase olive mill waste (85%) with fresh horse bedding (15%) –% by dry weight–. This compost was produced in a pilot plant (Centro de Edafología y Biología Aplicada del Segura, Murcia, Spain) by a dynamic composting system with periodical pile turning during the bio-oxidative phase (which lasted 195 days), followed by 66 days of maturation.

Physico-chemical parameters were determined in a 1:5 (v:v) water suspension or extract for pH and electrical conductivity (EC), respectively, as indicated by the European standards (EN 13037, 1999; EN 13038, 1999). S-SO_4^{2-} was extracted sequentially with a 0.15% $CaCl_2$ solution and determined gravimetrically following the

Clesceri et al. procedure [4], expressing the sulphate content as $CaSO_4$ (% dry wt). Carbonate contents (expressed as $CaCO_3$, % dry wt) was analysed by measuring the CO_2 released after the acidification of a compost sample using the Bernard's calcimeter according to the Spanish Ministry of Agriculture, Fisheries and Food [5].

Finally, the sample was serially diluted and microbial colonies were counted after incubation on several culture media: Medium 152 (American Type Culture Collection, ATCC) for autotrophic bacteria, including tiobacilli; Plate Count (Merck) for heterotrophic bacteria; Actinomycete Isolation Agar (Difco) for actinomycetes; and, Sabouraud Agar (Merck) for fungi.

2.2 Acification experiment

TPOMW compost was amended with the dose D (= 9.5 g S^0 L^{-1} compost) of powdered S^0 previously determined –from a titration experiment– to reach a pH value of 5.5, as described by Martínez et al. [6]. An acidification assay was then performed at 30ºC and constant compost humidity (equivalent to 80% of the container capacity) for 70 days. At selected intervals –0, 3, 9, 15, 23, 36, 50, and 70 days after the beginning of the assay– pH, EC, carbonate and sulphate contents as well as microbial populations were determined in representative samples by using the methods described in the section 2.1. The changes in the populations studied have been presented in comparison with (Δ) the un-amended control.

2.3 Isolation and identification of sulphur-oxidizing strains

From the culture medium ATCC 152 inoculated with the amended compost (section 2.2), several potential sulphur-oxidizing strains were isolated and characterized by using the catalase and oxidase biochemical tests as well as the Gram tinction and macroscopic morphology studies. On the basis of these tests, 5 presumptive sulphur-oxidizing bacteria were selected to perform their identification prior to a compost inoculation experiment (see section 2.4).

Total DNA was extracted according to Wilson [7] and subsequently it was subjected to PCR amplification using primers 27f and 1492r as described by Lane [8]; the PCR product was then purified by the GenElute Kit with the same sets of primers. The 16S rRNA gene sequences were obtained using the ABI Big Dye Terminator Cycle Sequencing Kit version 3.1 and the automatic sequencer Applied Biosystems 3730xl DNA Analyzer; they were assembled subsequently from the combination of separate fragments generated with forward and reverse sequencing primers by the PHYDIT software package, obtaining an almost complete sequence of each 16S rRNA gene. From these sequences, the identification was determined using the Basic Local Alignment Search Tool (BLAST) offered by the National Center for Biotechnology Information (NCBI).

2.4 Compost inoculation with selected bacterial strains and acidification efficiency

After the identification of the 5 selected strains, 3 different sulphur-oxidizing bacteria were used to inoculate sterilized compost amended with the dose D of S^0. In addition, this experiment was carried out with 3 reference strains of sulphur-oxidizing bacteria: *Thiobacillus denitrificans* 104767 CIP, *Thiomonas intermedia* 104401 CIP, and *Halothiobacillus neapolitanus* 104769 CIP. Inocula were added at a rate of 10^7 to 10^8 CFU g^{-1} sterilized compost; non-inoculated, sterile and non-sterile controls were also prepared. Inoculated materials were incubated for 18 days at 30ºC and constant humidity, and both pH and EC as well as the bacterial colonies mentioned above were monitored during this period. The results for each inoculated strain have been shown as maximum increase of pH, EC and sulphur-oxidizing bacteria throughout the assay. In addition, the microorganisms' ability to oxidize elemental sulphur was compared by calculating an acidification efficiency index (ae) [6]. Statistical analyses were performed using the Statgraphics Plus 5.1 statistical package.

3. Results and discussion

3.1 Compost characteristics

As shown in Table 1, TPOMW compost presented a high EC and a strongly alkaline pH, thus indicating that a pH reduction is required for its use as soil improver. The values for these parameters were similar to those obtained for composted two-phase olive mill residues in other studies [1,2]. Both the sulphate and carbonate contents were lower than the levels reported previously for other composts prepared with organic wastes from horticultural, farming and forestry sources [3]. Concerning the microbial population counts, the highest concentration applying to actinomycetes (8 logarithmical units), whereas autotrophic and heterotrophic bacteria showed an intermediate level and fungi the lowest concentration.

Table 1 Selected physico-chemical, chemical and microbiological characteristics of the compost studied.

Parameter	Value	SD
pH	9.17	0.02
Electrical conductivity (dS m^{-1})	3.65	0.08
Carbonates ($CaCO_3$, % dry wt)	10.8	0.07
Sulphates ($CaSO_4$, % dry wt)	0.41	0.03
Autotrophic bacteria (log CFU g^{-1})	7.38	0.05
Heterotrophic bacteria (log CFU g^{-1})	7.62	0.02
Actinomycetes (log CFU g^{-1})	8.25	0.03
Fungi (log CFU g^{-1})	6.20	0.01

3.2 Acification experiment. Isolation and identification of selected strains

The pH decrease measured during the acidification assay was higher than 3 units (achieving a final value of 6.1) and occurred simultaneously to the production of sulphates and the removal of carbonates (Figure 1). A great increase in EC also paralleled the pH drop. After the addition of S^0, autotrophic bacteria were enhanced remarkably, showing the largest increase on day 23 of incubation. By contrast, S^0 application did not affect significantly heterotrophic bacteria, actinomycetes' and fungi growth; only slight variations in these populations (lower than 0.5 logarithmical units) were recorded, thus suggesting that these microorganisms are not involved in the acidification of TPOMW compost.

In this experiment, a total amount of 31 potential sulphur-oxidizing strains were isolated and afterwards characterized (data not shown) to select 5 presumtive strains with the capability of oxidizing S^0. The identification obtained from 16S rRNA gene sequences analysis revealed that these selected strains were sulphur-oxidizing bacteria: 3 strains were identified as *Paracoccus thiocyanatus* (similarity of 99%), 1 as *Halothiobacillus neapolitanus* (similarity of 100%), and 1 as *Pseudomonas stutzeri* (similarity of 99%). These 3 different sulphur-oxidizing bacteria were used subsequently to inoculate the sterilized compost (see section 3.3).

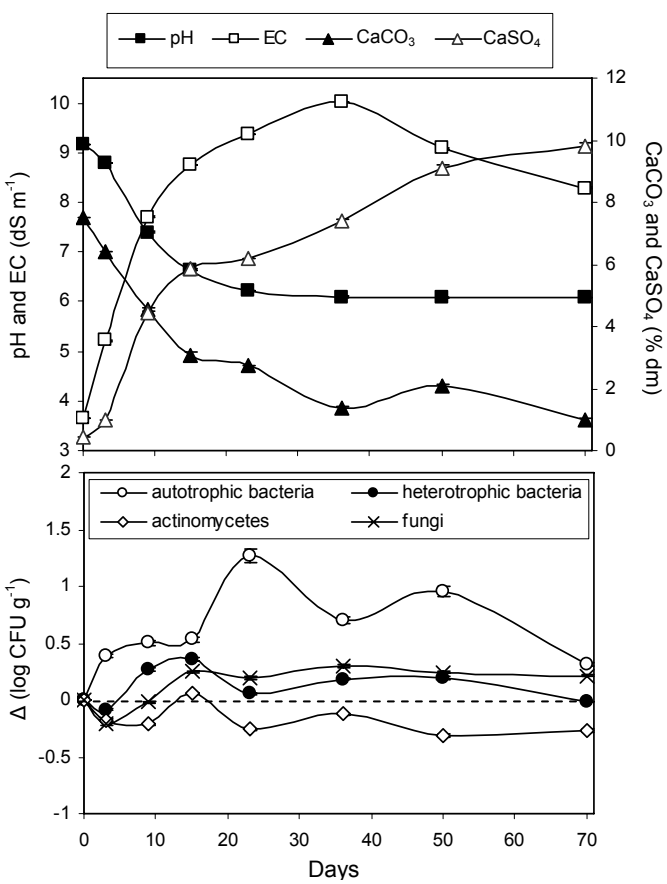

Fig. 1 Changes in pH, electrical conductivity (EC), carbonate and sulphate contents, and microbial populations throughout the acidification experiment after the addition of 9.5 g S^0 L^{-1}compost.

3.3 Acidification efficiency assessment of sulphur-oxidizing bacteria

Sterilized compost samples inoculated with any from the 3 identified sulphur-oxidizing strains or *Halothiobacillus neapolitanus* 104769 CIP showed a pH decrease (~2.4 units) and an acidification efficiency (from 75% to 81%) similar to those in the non-sterile compost, whereas a lower acidification efficiency (<60%) was observed when *Thiomonas intermedia* 104401 CIP or *Thiobacillus denitrificans* 104767 CIP were added (Table 2).

EC of inoculated, sterilized compost samples generally increased in parallel with the pH decrease and the sulphur-oxidizing bacteria population increase observed.

Table 2 Maximum increase in pH, electrical conductivity (EC) and sulphur-oxidizing bacteria population, and acidification efficiency index (ae) of sterilized and inoculated compost samples after 18 days of incubation.

Inoculum	ΔpH	ae (%)	ΔEC (dS m^{-1})	Δsulphur-oxidizing bacteria (log CFU g^{-1})
Non-sterilized control	-2.5a	77b	3.6a	3.2a
H. neapolitanus	-2.4b	78b	2.9bc	3.0a
P. thiocyanatus	-2.3c	75b	2.9bc	2.4b
P. stutzeri	-2.3c	75b	2.8c	1.4c
H. neapolitanus 104769 CIP	-2.5a	81a	3.0b	3.6a
T. intermedia 104401 CIP	-1.8d	58c	2.4d	2.1b
T. denitrificans 104767 CIP	-1.7e	55d	2.2e	1.1c
Significance[z]	***	***	***	***

[z] ***$P \leq 0.001$. Values within a column followed by the same letter do not differ significantly at $P \leq 0.05$ by LSD test

4. Conclusions

Taking into account the heterogeneity of genera which are capable of oxidizing elemental sulphur, the analysis of 16S rRNA gene sequences seems to be a rapid and effective method to identify sulphur-oxidizing bacteria.
Microorganisms which are native to the compost were associated with an acidification efficiency higher than reference strains which were not present in this organic material. Therefore, further research should focus on the improvement of the acidification efficiency by the addition of the isolated strains at different rates of inoculum, by adding a combination of multiple strains, or by recovering other sulphur-oxidizing strains from composts prepared with other organic wastes.

Acknowledgements This research was carried out within the framework of the projects PTR1995-0894-OP-02-02 and UPV-PPI-05-05-5542 subsidized by the Spanish Ministry of Education and Science (MEC) and the Polytechnic University of Valencia (UPV), respectively, as well as a PhD fellowship supported by the Valencian Council of Education.

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Manipulating the growth of bold and small grains in the ear of *Triticum aestivum* by salicylhydroxamic acid

Davood Eradatmand Asli*

Department of Agronomy and Plant Breeding, Islamic Azad University Saveh Branch, Saveh-Iran

*Corresponding e-mail address: eradatmand_d@yahoo.com

Earlier studies have shown that the two types of sinks- bold and small grains-developing in the same spike or spikelet of wheat (*Triticum aestivum* L.) had their inherent variable metabolic profiles, which were steered by their endogenous hormones. In the present investigation, it is being demonstrated that a check on electron transport chain during respiration led to the partial truncation of the dry matter of the developing grains. The study indicates that another subtle phenomenon, involving the feed off of the electrons to an alternate oxidase pathway, switches-on in vivo during the mid-ripening stage of the grains and may be detrimental to their growth. It is being advocated that the modulation of this pathway, through the judicious use of its specific inhibitor salicylhydroxamic acid may go a long way in achieving a higher economic yield. The data further imply that it may not be possible to eliminate the disparity between the two types of sinks in their yielding potential, which appears to be their innate character.

Keywords: CN-resistant respiration; inhibitor; regulation; sink efficiency; wheat

Introduction

A casual look into the present global food supply reveals that the cereals constitute 2/3rd component of its resource. An appraisal of parameters regulating their productivity divulges that their full potential to yield is still unrealized. One of the grey areas, which has remained untapped, is the host of physiological and genetical barriers of developing kernels to grow to an optima and their manipulation by desirable traits and methodologies. The potential upgradation of components constituting the total yield in wheat (number of productive tillers m^{-2}, grains per spike and 1000-grain weight), would help to raise the production substantially. Though, significant milestones have been achieved in the first two parameters the last component, the individual grain weight has eluded scientific investigations and rather paradoxically has declined with the advent of high yielding varieties. A study into the physiology of grain yield shows the existence of variation among different varieties or genotypes or even the grains developing in the same ear [1, 2, 3, 4]. It further discloses that the yield may be influenced by the availability of photosynthates to the developing sinks [5, 6, 7]. Various sugar responsive genes in plants potentially affect the partitioning [8] and have been stressed to be key determinant of plant productivity [9]. Dry matter partitioning also plays a paramount role in growth rate of sink organs [10]. Working on the grain growth in wheat and *Fagopyrum*, variation among varieties was traceable to endogenous hormone production in variety *vis-a-vis* that in the ear [11]. A few biochemical components as advocated by [12, 13] might be of significance in determining sink efficiency and/or the grain yield.

Since, the harvest index is the culmination of innumerable events, most of the view points on sink efficiency appears to be speculative and need a holistic approach in isolating obligatory events to produce the net assimilates. The revelation that the electron transport chain, in operation during biological oxidation, might find an alternate route without performing the target aim of creating proticity and may downgrade the overall impetus of meristems to grow by 10 to 25 per cent [2,3]. Indeed, it has been reported that higher alternative respiration could be one of the reasons of lesser growth of grains at distal position in a spikelet [2]. It is, therefore, advocated that any attempt to interrupt this process may prove beneficial in improving productivity.

In the present study, it is proposed to analyse the differential dry matter accumulation potential of different grains, growing in the same spikelet along with their behaviour under the influence of electron transport chain inhibitor. It is also aimed to modulate the alternate oxidase pathway through the use of specific inhibitor salicylhydroxamic acid (SHAM) and to find out the stage when grains respond to SHAM maximally.

Materials and methods

The investigations were conducted with common bread wheat *(Triticum aestivum* L. emend Thell var. PBW-34), which was sown on November 15, 2005 in circular earthenware pots (50x30x30 cm) containing 35 kg of soil mixed with farmyard manure (4:1). Eight seeds per pot were sown and after 15 days, seedlings were thinned to

two. Hoagland's nutrient solution [14] was supplied to the pots. Mother shoots, which formed the exclusive material for the studies, were tagged and anthesis date was recorded therein. Growth rate of different grains were recorded at intervals of 7 days, following anthesis until maturity, samples for dry weight, were taken by counting clock-wise the florets in the spikelets facing the upper side of flag leaf and occupying 6^{th}, 7^{th}, 8^{th} and 9^{th} spikelets (middle position of the spike) from the base of the ear. Calculations of the growth rate (GR), relative growth rate (RGR) were done employing the equation given by Evans *et al*. [15]. Antimycin A and salicylhydroxamic acid in two concentrations (1 and 10 ppm) were prepared. These inhibitors were applied at anthesis stage in five replications with the help of cotton plugs, which remained on ears of mother shoots (MS) for 48 hours. The data were analysed statistically using analysis of variance and critical differences at 5 per cent level were computed.

Results and discussion

The analysis of data on the growth patterns revealed that the grains occupying different positions in the same spikelet varied with regard to their potential to gather dry matter. The basal grains of constitutive spikelet showed higher dry matter accumulation than the one growing distally. The former was labeled as bold while the latter was tagged as small grain. The differentiality in their precipitation potential was correlated with their growth rates (Table 1). As evident from the Table 1, the smaller grains were conspicuous by relatively lower growth rates to the tune of 27, 25, 23, 56 and 14 per cent at 7, 14, 21, 28 days after anthesis and at maturity respectively. Relative growth rates (RGR) depicted an unusual behaviour. The important observation was that irrespective of the stage of grains growth, the RGR values in two types of the grains were not significantly different, thereby implying that the potentiality to grow amongst the two types of grains was of similar magnitude and its was the per se availability of the growing mass along with their initial capita which possibly maintained the disparity despite their equal potentials to grow.

The application of antimycin A significantly reduced the dry matter accumulation potential in both the types of grains at all the stages examined (Fig. 1). The analysis of the data revealed that the two types of grains showed variability in their response to inhibitor at early stages e.g., at 7 days the small grains suffered more severely with a net reduction of 95 and 96 per cent in dry weight in the two concentrations as compared to 56 and 70 per cent in bold grains at these respective concentrations. However, at 14, 21, 28 days post-anthesis stages, the severity in reduction was lesser, in the range of 45 to 65 per cent with no significant differences between bold and small grains. Similarly at harvest the grains showed a net reduction in range of 60 to 63 per cent with no significant difference between the small and bold grains. Interestingly, the two types of grains continued to show a disparity with regard to their total dry matter between them and at maturity the smaller grains had 35 and 23 per cent lesser net dry weight as compared to the bold grains under the influence of 1 and 10 ppm of antimycin A, respectively.

Table 1. Growth rate (mg/day) and relative growth rate (mg/mg/day) of individuals grains of *Triticum aestivum* L. var. PBW-34 isolated from different position at different intervals of time after anthesis.

DAA	Growth Rate		Relative Growth Rate	
	B	S	B	S
1-7	1.0	0.7 (-27.4)	0.14	0.14
7-14	1.5	1.2 (-25.7)	0.04	0.04
14-21	0.9	0.7 (-23.1)	0.05	0.05
21-28	1.6	0.7 (-56.5)	0.02	0.02
28-M	0.9	0.8 (-14.7)	-	-
CD (5%)				
Interaction:	0.26		0.28	
Position:	0.08		0.12	
Age:	0.25		0.27	

DAA=Days after anthesis, B=Bold grain, S=Small grain, M=maturity

The parallel set involving the application of salicylhydroxamic acid revealed the unique observations (Fig. 2). Ironically, the inhibitor behaved in an enigmatic way and proved to be a promoter when being assessed under the criterion of dry matter accumulation in grains. During the earlier period of grain development (7 days to 21 days) it had no significant effect thereby indicating that the underlying physiological process may not be in

operation. Subsequently a significant increase in dry matter accumulation in both the types of grains at 21 days onwards upto maturity was noticed at both the concentrations of SHAM. The highest increment was recorded at 21 days post-anthesis stage (51 and 49 per cents respectively for bold and small grains), whereas at maturity the enhancement in bolder and smaller grains was to the tune of 10 and 11 per cent at 1 ppm and 14 and 24 per cent at 10 ppm concentration, respectively.

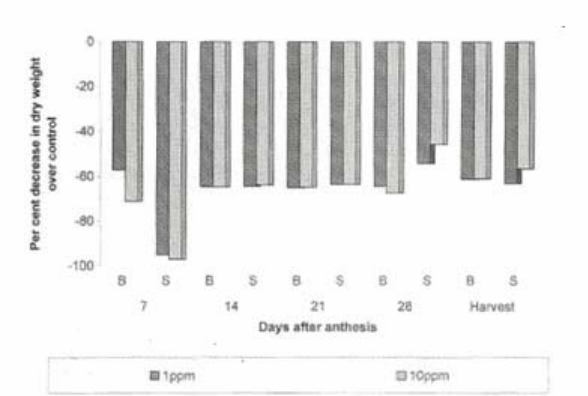

Fig. 1 Per cent decrease in dry weight of grains at different locations in wheat (*Triticum aestivum* L. var. PBW-34) under the influence of antimyein A.

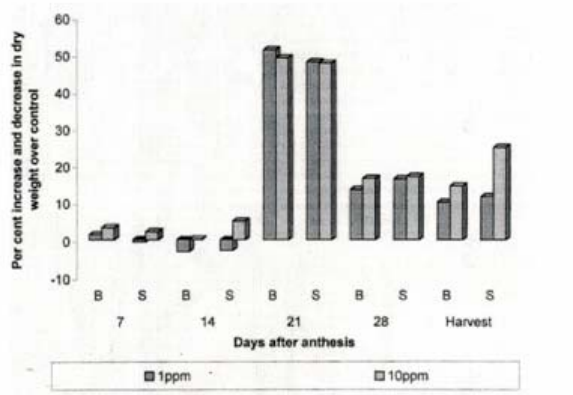

Fig. 2 Per cent increase or decrease in dry weight of grains at different locations in wheat (*Triticum aestivum* L. var. PBW-34) under the influence of salicylhydroxamic acid.

The salient points emerging through the use of salicylhydroxamic acid were that (i) grains (both bold and small) showed a significant increase in dry matter from 21 days post-anthesis stage with its applications and (ii) in spite of the aforementioned increment gathered by grains, they continued to exhibit the disparity between them and at maturity the smaller grains still showed 30 and 25 per cent lesser dry matter than the bolder grains.

The results bring forth, in no uncertain terms, the findings that the ear of wheat is a developing place for a definite number of grains which inturn are separate biological entities endowed with their inherent potentials to grow and accumulate dry matter. This axiom was advocated by Abolina [16] and is in line with the observations of innumerable workers [3, 4, 17, 18]. Nevertheless, the sequence of events, piloting the yielding ability, is the metabolic profile and if augmented through the use of plant growth regulators or by imposing a shift in metabolic events promotery effects are achievable [19, 20]. In the present context, the contention is further testified that when the inhibitor of respiration intercepted this process there was a corresponding decrease in the net weight of the grains, irrespective of their parchment in the inflorescence. However, the central point which came to light in the present endeavour is that an unusual path of aerobic respiratory chain (CN-resistant respiration) plausibly switches-on during the mid-maturity stages (14-21 days after anthesis) and if checked, through the immaculate use of salicylhydroxamic acid, can significantly increase the dry matter accumulation potential of the grains. Of course, SHAM (regulator of alternative oxidative pathway) was not successful in eliminating the disparities between the two types of grains. The present work concludes that amongst a plethora of metabolic passes and bypasses an appraisal of the alternate oxidase pathway needs to be assessed and appraised as advocated by [21,22] and its regulation would definitely go a long way in improving grain yield in different cereals specially wheat.

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New hosts for the enterobacterial phytopathogen *Erwinia persicina*

Diánez F., Santos M.*, Font I., de Cara M., Tello J.C.

Dpto. Vegetal Production. University of Almería. 04120. Spain

*Corresponding author: msantos@ual.es

The aim of this paper was to search for alternative hosts to green bean for *Erwinia persicina.* This enterobacteria, detected for the first time in southeastern Spain at the end of 2003, causes chlorotic and necrotic spots on leaves and pod deformation in green bean. Results showed that *E. persicina* caused disease in tomato, pepper, melon and cucumber. The most characteristic symptoms observed were: leaf necrosis, adventitious roots, brown colouring along the stem, internerval chlorosis, and curled and blistered leaves. In the case of cucurbits, a noticeable lesion in some areas of the stem was also observed. The presence of the bacteria in each plant was detected by microbiological and immunological analyses (ELISA).

Keywords: enterobacterial phytopathogen; hosts; symptoms.

Introduction

The genus *Erwinia* is classified in the Enterobacteriaceae family. Most members of this genus characteristically cause diseases in plants, vegetables and fruit. There is not much information about *Erwinia persicina* since it was described in 1990 after being isolated from a variety of fruit and vegetables, including bananas, cucumbers, and tomatoes in Japan [5]. *E. persicina* has been isolated from bean in the United Status [9] and Brenner et al. [1] reported that *E. persicina* (*E. persicinus*) was a senior subjective synonym for *E nulandii*, a pathogenic organism to bean pods and seeds. The phytopathogen *E. persicina* has been isolated from human urinary tract infections [8].

A previously unreported leaf spot disease of the common bean, which caused crop losses as high as 50 per cent, was observed in southeastern Spain (Almeria, Granada, and Malaga provinces) in November 2003. In 2004, samples of cv. Donna with chlorotic and necrotic leaf spots were collected from Granada and processed for microbiological analysis. Bacteria isolated from the symptomatic leaves were determined to be fermentative on the basis of their ability to metabolize glucose under aerobic and anaerobic conditions. The pathogen was identified by sequencing the gene encoding the ARN ribosomal 16S (ADNr 16S). The sequence obtained was compared with those registered in data banks and the largest percentage of homology (99%) was obtained to *Erwinia persicina*'s ADNr 16S. Therefore the bacteria causing this pathology could be clearly assigned to this species [3].

Many aspects relative to the virulence of *E. persicina* are unknown. Toxin production has not yet been described, contrary to what occurs in other phytopathogenic bacteria. Iron capture systems represent a strong candidate virulence factor [2].

Given the scarcity of existing references on the pathogenesis of this bacterium, our main objective was to determine the pathogenic capacity of Spanish *E. persicina* isolates in various plant species and to characterise their symptoms. Isolates were obtained from samples of cv. Donna with chlorotic and necrotic leaf spots collected in Granada (Spain) [3].

Materials and Methods

E. persicina inoculation

Bacterial suspensions (10^8 CFU/ml) were spray-inoculated on seedlings with 1-2 true leaves. The underside of previously perforated and non-perforated tomato, cucumber, melon and pea seedling leaves was sprayed with the bacterial suspension. Plants were covered with transparent plastic bags for 2 days and maintained in an incubation chamber at 25°C and 80% relative humidity with a 16/8-h photoperiod. Then the bags were removed and symptoms were observed for 50 days. [3]. Plants in the control group, inoculated with sterile distilled water, showed no symptoms. The bacterium was readily re-isolated from diseased leaves. Assays were repeated at least twice.

E. persicina diagnosis

Isolation was conducted through techniques proposed by Lelliot & Stead [7]. Leaves were washed with water, then exteriorly disinfected with 70% alcohol for one minute and washed again with sterile distilled water. Affected pieces were macerated in a mortar with sterile distilled water. The macerate was streaked onto King's B medium [8], incubated in the dark at 25ºC for 48 hours and consecutively tapped to obtain pure bacteria colonies. Diagnosis was also made by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

Results and Discussion

Samples were analysed by isolating bacteria from diseased plants as well as through the DAS-ELISA technique. All the diseased bean plants, from which *E. persicina* was isolated, corroborated the symptoms observed in the plants and described in previous studies [8] such as internerval chlorosis, leaf necrosis and the appearance of adventitious roots in the stem. Besides, previously undescribed symptoms were observed: the appearance of necrotic spots on the underside of the leaves as well as deformation, curling and marked blistering (Fig. 1). All plants with these symptoms were positive in enzyme-linked immunosorbent assay (ELISA) tests using *E. persicina* antiserum. The plants that tested negative for the presence of *Erwinia persicina* with the DAS-ELISA technique could represent an error in carrying out the technique or quite probably, those two bean plants were already rather dry when the samples were taken. Generalized necrosis was observed in pea, described recently by Gonzalez et al. [4] in *Pisum sativum* cv. Tirabeque. In addition, the appearance of a shiny brown necrosis was observed on the underside of the sprayed leaves which was not detected in newly emerging leaves. Necrosis was also described as beginning on the sides of the leaf and later advancing towards the centre. Chlorotic symptoms were not observed at any time.

The appearance of symptoms in cucumber plants could also be correlated with the presence of *E. persicina.* The main symptoms observed in cucumber were: necrosis in basal leaves, blistering of leaves, drooping of leaves, conspicuous stem lesions and late appearance of internerval chlorosis (Fig. 2).

The first symptoms were observed in tomato plants one week after being inoculated with the bacteria. These symptoms were tiny purple spots on the stems and leaves and silver necrosis on the underside of the leaves. Two weeks after inoculation the appearance of adventitious roots was observed along the entire stem with secondary ramifications. Lastly, a new symptom was observed in several of the inoculated plants: internerval leaf chlorosis, especially in plants with microwounds (Fig. 3).

Silver leaf necrosis was observed in melon five days alter inoculation, although not in every plant. Later an inward curling of leaves was seen and adventitious roots all along the stem, especially at the base. At first these symptoms were quite scarce, barely developed and did not appear in every plant. Lastly, blistering of leaves, internerval leaf chlorosis and stem lesions were seen (Fig. 4). The presence of this phytopathogenic bacterium had never been described in this species; therefore, the symptoms observed could not be contrasted with other references.

For all the plant species under study, significant differences were not observed between inoculation methods, either perforated or non-perforated leaves, or in the time of symptom appearance or the various symptom types. Analyses of symptomatic plants from the various plant species under study confirmed the presence of *Erwinia persicina.* Likewise, symptoms by Gonzalez et al. caused by this bacteria in bean and pea crops were confirmed [3][4]. In addition, the pathogenic capacity of *E. persicina* was described for the first time in melon under controlled conditions.

Figure 1. Symptoms caused by *E. persicina* in bean

Figure 2. Symptoms caused by *E. persicina* in cucumber

Figure 3. Symptoms caused by *E. persicina* in tomato

Figure 4. Symptoms caused by *E. persicina* in melon

Acknowledgments This work was sponsored by project RTA2006-00033-C03-02 from the Ministry of Education and Science-INIA.

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Production and Evaluation of Polyclonal Antisera for Detection of *Ralstonia solanacearum*

Nader El-Badry*, Safwat El-Haddad, Ahmed Hussien

Potato Brown Rot Project, Dokki, Giza, Egypt

*Corresponding author: e-mail: nader.el-badry@alumni2005.ncl.ac.uk, Phone: +20-2-37498673

Polyclonal antisera have been developed against *Ralstonia solanacearum* bacterium, the causal organism for brown rot in potato plants, and have been evaluated at Agriculture Research Centre (ARC), Potato Brown Rot Project (PBRP), Dokki, Giza, Egypt. The following antigenic mixtures; fixed whole cells, soluble cell components, cell washings (exopolysaccharides) and heat-stable cell components have been used to produce antisera from rabbits. Results have shown that antigenic mixtures have significant effect on specificity and sensitivity of antisera. The immunofluorescence antibody staining (IFAS) has been used to determine titre, specificity and sensitivity of each poduced antiserum. Different antisera have showed variance in their titre level whether for those produced after 6 or 12 injections or at death.

Keywords Antisera, *Ralstonia solanacearum*, Brown rot, Potato, Immunofluorescence antibody staining test, Titer.

1. Introduction

One of the greatest difficulties in the control of brown rot has been the absence of an efficient and easily used method for routine, rapid and accurate detection of *R. solanacearum* in large numbers of plant, soil and water samples.

Serological techniques are good compromise between sensitivity and specificity of detection, and ease, and expense of application.

Several methods have been proposed for the use of polyclonal antibodies (PAbs) in the detection of *R. solanacearum*, the immunofluorescent technique has been the most widely used, despite it being both laborious and relatively costly. The method has been recommended by European Union (EU) as first screening test (Janse, 1988).

Immunofluorescence Antibody Staining (IFAS) test is a key screening test that involves staining the target bacterial cells with specific antibodies to which are bound fluorescent markers. Selective binding of the antibodies to the bacterial cell walls allows them to be observed under UV microscopy.

IFAS test is widely used in EU for detection of bacterial pathogens in plant materials for the presence of *R. solanacearum* and *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of ring rot disease (Elphinstone, 2004). It is also used as the principal brown rot screening test in Egypt (Farag, 1999). In the Netherlands, IFAS is used to screen 60,000 seed potato samples annually. IFAS is also used to detect many other bacterial pathogens, for example in France it is used to screen tomato seed lots for the bacterial canker pathogen, *C. michiganensis* subsp. *michiganensis*.

2. Materials and methods

2.1. Bacterial isolates and antigen preparation

One reference bacterial isolate of *R. solanacearum* was cultured in 9 cm plastic Petri dishes on TZC medium but without tetrazolium (Kelman, 1954), and incubated at 28^{o}C for 24-28 h. All other bacterial isolates (Table 1) were cultured on nutrient glucose agar (NGA) media. *R. solanacearum* cells were harvested in sterile phosphate buffer saline (PBS) 10mM, pH 7.2 (1L distill water, 8gm NaCl, 0.4gm $NaH_2PO_4.2H_2O$ and 2.7gm $NaHPO_4.12H_2O$). Four antigenic mixtures were prepared 1- Cell washings (exopolysaccharides) components, 2- Heat resistant antigens (boiled cells), 3- Whole fixed cells, and 4- Soluble proteins.

2.2. PAb production

Eight New Zealand white male rabbits were immunized with 12-13 intramuscularly injections; except the first injection was subcutaneously. The rabbits were injected with increasing dosages of the antigens mixed with Freund's incomplete adjuvant (Sigma, Germany) 1 adjuvant: 1 inoculum (v : v) (see immunization schedules) as following: Two rabbits were injected with cells washings (extracellular-polysaccharides), two rabbits with fixed cells by glutaraldehyde, two rabbits were immunized with dry heated cells, and two rabbits with soluble cell components. As for cells washings, bacterial cells were centrifuged for 15 min at 14500 ×*g* and then were filtered by Millipore filters of size 0.25µl and the clear supernatants used for the immunization. The boiled cells were broken down by dry heating at 99°C for 10 min then incubated on ice for 15 min and stored at -30°C for the immunization. Fixed cells were adjusted at a concentration of 5×10^8 cells/ml^{-1} after killing and fixation by glutaraldehyde (Robinson-Smith *et al.*, 1995). Soluble cell components was prepared by breaking the bacterial cells by ultrasound then centrifuged for 15 min at 14500 ×*g* and supernatant was used then estimated to the proteins according to Robinson-Smith (Robinson-Smith *et al.*, 1995).

All rabbits were injected 12-13 times, then bleeding was carried out for all of them two times and the third time was to collect their blood after slaughtering (death). Blood was collected from the lateral ear vein four weeks after the first immunization and after the eighth injection then the last blood sample was collected after slaughtering. The blood was allowed to clot at room temperature, separated by centrifugation (1500 ×*g* for 15 min) and the serum fraction was collected (A. Robinson-Smith, *et al*., 1995).

Table 1 Bacterial isolates used in this study

Bacterial isolate	Source
Ralstonia solanacearum	Potato tuber
Acinetobacter Sp.	*PBRP collection
Pseudomonas fluorescence	PBRP collection
Escherichia coli	PBRP collection
Clavibacter michiganensis subsp. *sepedonicus*	PD collections, Netherlands
Bacillus thuringiensis	PBRP collection

***PBRP collection**: Potato Brown Rot Project bacterial collection

2.3. Immunization schedule

Rabbit 1

Soluble proteins

Injection	Date	Dose	Injection type	Titre
Inj. 1	27/Aug./05	0.5 mg	Subcutaneous injection	
Inj. 2	03/Sep./05	0.5 mg	Intramuscularly	
Inj. 3	10/Sep./05	0.75 mg	Intramuscularly	
Inj. 4	17/Sep./05	1 mg	Intramuscularly	
Inj. 5	24/Sep./05	1 mg	Intramuscularly	
Inj. 6	02/Oct./05	1.25 mg	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:3000
Inj. 7	10/Oct./05	1.5 mg	Intramuscularly	
Inj. 8	17/Oct./05	2.5 mg	Intramuscularly	
Inj. 9	24/Oct./05	3 mg	Intramuscularly	
-	31/Oct./05	-	Delay of injection	
Inj. 10	07/Nov./05	3.125 mg	Intramuscularly	
Inj. 11	13/Nov./05	3.5 mg	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:4000
Inj. 12	21/Nov./05	4 mg	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:3000

Rabbit 2

Whole fixed cells (glutaraldehyde)

Injection	Date	Dose	Injection type	Titre
Inj. 1	27/Aug./05	1×10^{8}	Subcutaneous injection	
Inj. 2	03/Sep./05	1×10^{8}	Intramuscularly	
Inj. 3	10/Sep./05	2×10^{8}	Intramuscularly	
Inj. 4	17/Sep./05	3×10^{8}	Intramuscularly	
Inj. 5	24/Sep./05	3×10^{8}	Intramuscularly	
Inj. 6	02/Oct./05	4×10^{8}	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:3000
Inj. 7	10/Oct./05	5×10^{8}	Intramuscularly	
-	17/Oct./05	-	Delay of injection for medical reasons	
Inj. 8	24/Oct./05	1×10^{9}	Intramuscularly	
Inj. 9	31/Oct./05	2×10^{9}	Intramuscularly	
Inj. 10	07/Nov./05	2×10^{9}	Intramuscularly	
Inj. 11	13/Nov./05	2×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:3000
Inj. 12	21/Nov./05	2.5×10^{9}	Intramuscularly	
-	16/Nov./05	-	Died before slaughtering	

Rabbit 3

Polysaccharide

Injection	Date	Dose	Injection type	Titre
Inj. 1	03/Sep./05	4×10^{8}	Subcutaneous injection	
Inj. 2	10/Sep./05	4×10^{8}	Intramuscularly	
Inj. 3	17/Sep./05	8×10^{8}	Intramuscularly	
Inj. 4	24/Sep./05	8×10^{8}	Intramuscularly	
Inj. 5	2/Oct./05	1.2×10^{9}	Intramuscularly	
Inj. 6	10/Oct./05	1.6×10^{9}	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:3000
Inj. 7	17/Oct./05	2×10^{9}	Intramuscularly	
Inj. 8	24/Oct./05	2×10^{9}	Intramuscularly	
Inj. 9	31/Oct./05	4×10^{9}	Intramuscularly	
Inj. 10	07/Nov./05	4×10^{9}	Intramuscularly	
Inj. 11	13/Nov./05	6×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:3000
Inj. 12	21/Nov./05	8×10^{9}	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:3000

Rabbit 4

Heat resistant antigens (boiled cells)

Injection	Date	Dose	Injection type	Titre
Inj. 1	27/Aug./05	1×10^{8}	Subcutaneous injection	
Inj. 2	03/Sep./05	1×10^{8}	Intramuscularly	
Inj. 3	10/Sep./05	2×10^{8}	Intramuscularly	
Inj. 4	17/Sep./05	3×10^{8}	Intramuscularly	
Inj. 5	24/Sep./05	3×10^{8}	Intramuscularly	
Inj. 6	2/Oct./05	4×10^{8}	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:3000
Inj. 7	10/Oct./05	5×10^{8}	Intramuscularly	
Inj. 8	17/Oct./05	1×10^{9}	Intramuscularly	
Inj. 9	24/Oct./05	2×10^{9}	Intramuscularly	
Inj. 10	31/Oct./05	2×10^{9}	Intramuscularly	
Inj. 11	07/Nov./05	2×10^{9}	Intramuscularly	
Inj. 12	13/Nov./05	2×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:4000
Inj. 13	21/Nov./05	2.5×10^{9}	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:4000

Rabbit 5

Polysaccharide

Injection	Date	Dose	Injection type	Titre
Inj. 1	03/Sep./05	4×10^{8}	Subcutaneous injection	
Inj. 2	10/Sep./05	4×10^{8}	Intramuscularly	
Inj. 3	17/Sep./05	8×10^{8}	Intramuscularly	
Inj. 4	24/Sep./05	8×10^{8}	Intramuscularly	
Inj. 5	2/Oct./05	1.2×10^{9}	Intramuscularly	
Inj. 6	10/Oct./05	1.6×10^{9}	Intramuscularly	
-	17/Oct./05	-	First test-bleeding	1:3000
Inj. 7	17/Oct./05	2×10^{9}	Intramuscularly	
Inj. 8	24/Oct./05	2×10^{9}	Intramuscularly	
Inj. 9	31/Oct./05	4×10^{9}	Intramuscularly	
Inj. 10	07/Nov./05	4×10^{9}	Intramuscularly	
Inj. 11	13/Nov./05	6×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:3000
Inj. 12	21/Nov./05	8×10^{9}	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:3000

Rabbit 9

Heat resistant antigens (boiled cells)

Injection	Date	Dose	Injection type	Titre
Inj. 1	27/Aug./05	1×10^{8}	Subcutaneous injection	
Inj. 2	03/Sep./05	1×10^{8}	Intramuscularly	
Inj. 3	10/Sep./05	2×10^{8}	Intramuscularly	
Inj. 4	17/Sep./05	3×10^{8}	Intramuscularly	
Inj. 5	24/Sep./05	3×10^{8}	Intramuscularly	
Inj. 6	2/Oct./05	4×10^{8}	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:3000
Inj. 7	10/Oct./05	5×10^{8}	Intramuscularly	
Inj. 8	17/Oct./05	1×10^{9}	Intramuscularly	
Inj. 9	24/Oct./05	2×10^{9}	Intramuscularly	
Inj. 10	31/Oct./05	2×10^{9}	Intramuscularly	
Inj. 11	07/Nov./05	2×10^{9}	Intramuscularly	
Inj. 12	13/Nov./05	2×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:4000
Inj. 13	21/Nov./05	2.5×10^{9}	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:3000

Rabbit 10

Whole fixed cells (glutaraldehyde)

Injection	Date	Dose	Injection type	Titre
Inj. 1	27/Aug./05	1×10^{8}	Subcutaneous injection	
Inj. 2	03/Sep./05	1×10^{8}	Intramuscularly	
Inj. 3	10/Sep./05	2×10^{8}	Intramuscularly	
Inj. 4	17/Sep./05	3×10^{8}	Intramuscularly	
Inj. 5	24/Sep./05	3×10^{8}	Intramuscularly	
Inj. 6	2/Oct./05	4×10^{8}	Intramuscularly	
-	10/Oct./05	-	First test-bleeding	1:4000
Inj. 7	10/Oct./05	5×10^{8}	Intramuscularly	
Inj. 8	17/Oct./05	1×10^{9}	Intramuscularly	
Inj. 9	24/Oct./05	2×10^{9}	Intramuscularly	
Inj. 10	31/Oct./05	2×10^{9}	Intramuscularly	
Inj. 11	07/Nov./05	2×10^{9}	Intramuscularly	
Inj. 12	13/Nov./05	2.5×10^{9}	Intramuscularly	
-	21/Nov./05	-	Second test-bleeding	1:4000
Inj. 13	21/Nov./05	3×10^{9}	Intramuscularly	
-	30/Nov./05	-	Slaughtering (death)	1:3000

3. Results and discussion

Titer determination of PAbs

All PAbs had high titers and reacted with all *R. solanacearum* isolates when tested IFAS. Titer was determined for all antisera, soluble proteins, and whole fixed cells and they gave titers as high as 1:4000. However, the one was produced against heat resistant cells gave titer as high as 1:3000.

Specificity of PAbs for detection of *R. solanacearum* in IFAS

All of the produced antisera were prone to false positive reactions when used in neat concentrations but this was eliminated when used at the optimum working dilutions (the titre) as no cross-reactivity had been recorded except with *E. coli* or *B. thuringiensis*. It was easy to be distinguished among *R. solanacearum* cells, as *E. coli* cells were smaller than *R. solanacearum* cells or observed as completely rounded. In contrast, *B. thuringiensis* cells were longer than *R. solanacearum* cells. These species of bacteria are not pathogenic to potato nor present in or on potato plants, therefore the probability of confusion with them while examining potato samples is low.

Sensitivity of detection of *R. solanacearum* by PAbs in IFAS

The lowest concentration of *R. solanacearum* bacteria detectable by all PAbs when used at working dilutions was 1×10^4 cells ml^{-1}.

Acknowledgment This research was carried out at Potato Brown Rot Project, Dokki, Giza, Egypt and we acknowledge Ahmed Besheer, Muhammad Mahmoud and Abd Allah for their scientific support.

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Survival of *Erwinia amylovora* in rain water at low temperatures

E.G. Biosca[1,*] , R.D. Santander[1], M. Ordax[2], E. Marco-Noales[2], B. Águila[1], A. Flores[3] and M.M. López[2]

[1] Universidad de Valencia, Departamento de Microbiología y Ecología, Avenida Dr. Moliner 50, 46100, Burjassot, Valencia, Spain.
[2] Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, Carretera Moncada – Náquera, km 4.5, 46113, Moncada,Valencia, Spain.
[3] Universidad de Valencia, Servicio Central de Soporte a la Investigación Experimental, Avenida Dr. Moliner 50, 46100, Burjassot, Valencia, Spain.

[*]Corresponding author: e-mail: elena.biosca@uv.es, Phone: +34 96 354 31 94

Erwinia amylovora, causal agent of fire blight in rosaceous plants, is responsible of serious economic losses worldwide. Its survival in aquatic habitats was practically unknown until recent studies showed that it may survive in sterile natural water, with trace nutrients and low temperature enhancing this survival. However, little is known about the possible persistence of this pathogen in natural water in the presence of other microorganisms. The survival and pathogenicity of *E. amylovora* in rain water microcosms at low temperature has been monitored, using sterile rain water and warm temperature as controls. This bacterium was able to survive and remain pathogenic in rain water, being its survival favoured at low temperatures, probably by slowing down the influence of starvation and water microbiota. The present results demonstrate that the risk of *E. amylovora* waterborne transmission exists, raising new questions on the role of water in epidemiology and control of fire blight.

Keywords fire blight; survival; cold incubation; water microbiota; pathogenicity; water transmission.

1. Introduction

Erwinia amylovora, causal agent of fire blight in rosaceous plants, is a quarantine bacterium in the European Union, responsible of serious economic losses worldwide. The ability of this pathogen to survive and spread in different ways has been related to the difficult management of this disease [1], but the information about *E. amylovora* biology outside susceptible plants is still scarce. In spite of the numerous studies about the fire blight pathogen, its survival in aquatic environments is practically unknown, as well as the real importance of this possible reservoir in the spread of this bacterium. One major limitation of aquatic environments is nutrient deprivation, which stimulates a starvation response in diverse nonsporulating bacteria allowing their existence in a culturable state [2]. This stress has also been involved in the induction of the viable but nonculturable (VBNC) state [3] in which viable bacteria become incapable to grow on solid nonselective media. In oligothrophic conditions common in natural waters *E. amylovora* populations exhibit both survival strategies: a subpopulation experiences a starvation–survival response, while other enters into the VBNC state [4, 5]. Both mechanisms permit the survival of this pathogen in sterile natural water for long periods, maintaining its pathogenicity [4,5].

Other prevailing stress factor of water ecosystems is low temperature. It is known that incubation at 5ºC generally stimulates the entry into the VBNC state in many bacteria, including plant pathogens [6, 7, 8]. A negative influence of cold incubation in saline solution has been also reported for the fire bligh pathogen [9]. But we have recently shown that *E. amylovora* survival in sterile natural water was more favourable at 5ºC than at 26ºC [4], suggesting that low temperature may be a key factor in its survival in oligothrophic environments. However, the influence of cold incubation on the persistence of this pathogen in natural water in the presence of other microorganisms is still unknown. In this work, the survival and pathogenicity of *E. amylovora* in nonsterile rain water at low temperature have been investigated, using sterile rain water and warm temperature as controls.

2. Materials and Methods

2.1 Bacterial strain and water microcosms

The French reference strain of *E. amylovora*, CFBP-1430 (CFBP, Collection Française de Bactéries Phytopathogènes), was used in this study. Rain water microcosms were prepared with freshly collected rain water, using sterile water microcosms as control. Both types of microcosms were inoculated with 10^7 cfu/ml of

E. amylovora strain CFBP-1430, as previously described [4]. One half of these two types of microcosms was incubated at 5ºC, while the other half at 26ºC, as control, for a period of 45 days.

2.2 Enumeration of bacterial cells

The survival of *E. amylovora* strain CFBP-1430 in sterile and nonsterile rain water microcosms at 5ºC and 26ºC was monitored by periodical enumeration of total, viable and culturable cells throughout 45 days. Plate counts were made on King's B medium [10], and total and viable counts were determined using the Live/Dead kit (Molecular Probes Inc., Eugene, Oreg.) with an epifluorescence microscope.

2.3 Pathogenicity tests

After 45 days of incubation in sterile and nonsterile rain water microcosms at 5ºC and 26ºC, *E. amylovora* cells of strain CFBP-1430 were inoculated on immature pear fruits (var. Williams) as previously reported [11]. Cells of strain CFBP-1430 from King's B solid medium and sterile PBS were used as positive and negative control, respectively. Symptoms development at 26ºC was recorded daily along 2 weeks.

3. Results

3.1 Survival of *E. amylovora* in rain water at low temperatures

One representative survival curve of the population dynamics of *E. amylovora* strain CFBP 1430 in sterile and nonsterile rain water microcosms at 5ºC and 26ºC is shown in Fig. 1. In both types of microcosms and at both temperatures, total and viable *E. amylovora* densities were similar to the initial ones, about 10^8 and 10^7cells/ml, respectively. On the contrary, different trends in the culturable population were observed depending on whether rain water was sterile or not and incubation temperature was 5ºC or 26ºC. In control microcosms with sterile water at 5ºC, there was a very slight decrease in the culturable counts from 10^7 to 10^6 cfu/ml within the first two weeks. Afterwards, culturable numbers were maintained to the end of the experiment. At 26ºC, a marked decline in culturable counts was observed after 15 days of incubation, from 10^7 to 10^5 cfu/ml, this decrease continued to cell densities about 10^4 cfu/ml at the end of the experimental period. In nonsterile water microcosms, containing rain water microbiota, the declines were more pronounced at both temperatures. At 5ºC, an initial decrease in the culturable cell counts from 10^7 to over 10^5 cfu/ml was observed for the first two weeks, remaning at these densities for the following weeks. At 26ºC, there was again a decline in plate counts more pronounced than at 5ºC, from 10^7 to 10^4 cfu/ml, with a continuous decrease with time up to 10^3 cfu/ml to the end of the experiment. In general, *E. amylovora* culturable cell numbers were two logarithmic units lower in the presence of rain water microorganims and when incubated at 26ºC.

Plate counts of rain water native bacteria were over 10^1 cfu/ml, being 10^2 cfu/ml in some water samples. Some of these bacteria, initially present in rain water, increased their numbers during incubation of nonsterile water microcosms, being these increases concurrent with the declines in *E. amylovora* plate counts (data not shown). They grew faster than the *E. amylovora* inoculated strain on King's B agar.

3.2 Pathogenicity of *E. amylovora* in rain water at low temperatures

E. amylovora cells from both sterile and nonsterile rain water microcosms at 5ºC and 26ºC were infective for immature pears after 45 days of incubation, causing necrosis and exudates in the inoculated fruits from where the pathogen was recovered and identified [11].

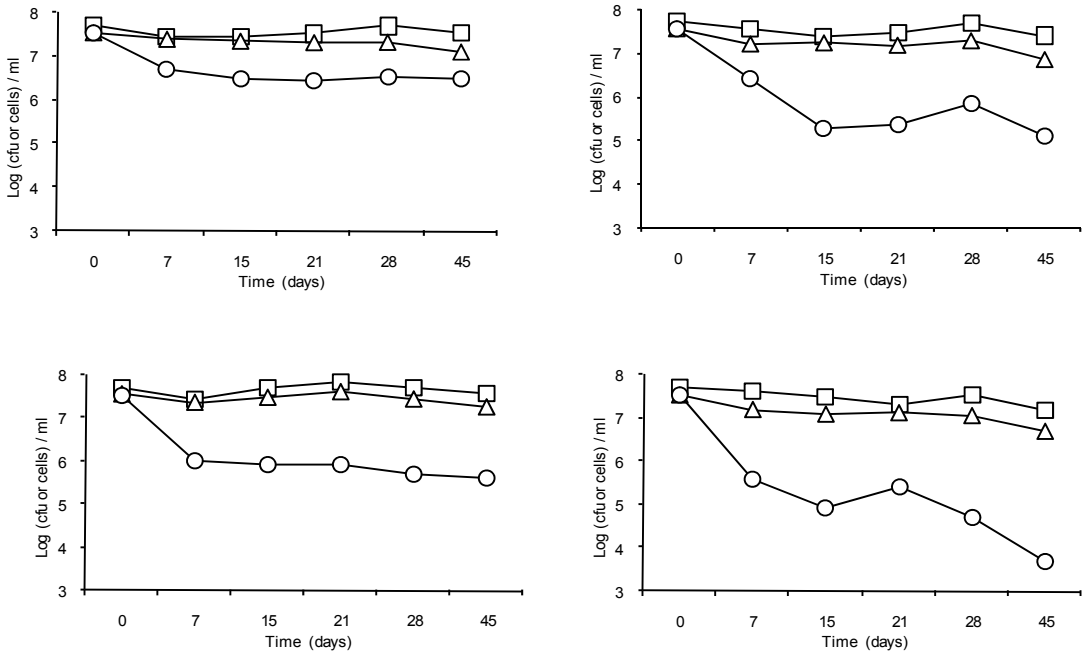

Fig. 1 Survival curves of *E. amylovora* strain CFBP-1430 in sterile (upper graphs) and nonsterile (lower graphs) rain water microcosms maintained at 5 ºC (left graphs) and 26ºC (right graphs): ○, plate counts on King's B medium; Δ, viable cell counts and □, total cell counts.

4. Discussion

We have previously demonstrated that *E. amylovora* may survive in distilled, drinking, rain and irrigation sterile waters for variable periods of time, showing that water can be a dissemination way for this pathogen [4, 5]. This survival was prolonged in the presence of trace water nutrients and by cold incubation [4]. However, the persistence of phytopathogenic bacteria in environmental waters can also be affected by aquatic microorganisms [8, 12, 13, 14], being their effects slower at lower temperatures [12]. To investigate the survival of *E. amylovora* in natural water at low temperatures, we decided to assay firstly rain water since it has been related with fire blight transmission [1], and low levels of water microorganisms were expected. The results from this work have shown that *E. amylovora* can survive and remain pathogenic in nonsterile natural rain water at least for 45 days, being its persistence more favourable at 5ºC than at 26ºC.

In control microcosms with sterile rain water, at both temperatures, total and viable *E. amylovora* densities were maintained over the experimental period. However, a decline was observed in culturable numbers, meaning that a fraction of the viable population entered into the VBNC state in response to the scarcity of nutrients. These results agree with our previous studies using different sterile waters [4, 5] and with others in some plant bacteria in oligotrophic conditions [15, 16]. Since the decline in culturable numbers was more pronounced at 26ºC than at 5ºC, as shown in sterile irrigation water [4], it seems clear that the induction of VBNC state can be more related with nutrient starvation than with low temperature. This supports the hypothesis that the overwintering of *E. amylovora* may be more related to the scarce plant nutrients during host dormancy [17] than to the lower temperatures common in winter.

Similar densities of total and viable *E. amylovora* populations were also observed in nonsterile rain water microcosms, at the two temperatures, also when compared to those in sterile water, suggesting that protozoa and/or virus, if present, would not affect substantially the survival of *E. amylovora* in the rain waters assayed. However, decreases in culturable counts were more pronounced in nonsterile water, being concurrent with increases in plate counts of some rain water native bacteria, which grew faster than *E. amylovora* on agar plates. Therefore, the survival of this pathogen in rain water could also be influenced by bacterial competition for scarce nutrients, despite other biotic interactions can not be excluded. The impact of native microbiota on the fate of other plant pathogens in water has been previously described [8, 12, 13, 14]. Further, the stronger and gradually increasing decline of *E. amylovora* plate counts at 26ºC than at 5ºC indicates a higher survival in natural water in cold conditions, probably because biotic effects were slower at lower temperatures, as reported for other important plant pathogenic bacteria [12].

Inoculation assays on pear fruits of *E. amylovora* cells from nonsterile rain water microcosms at 5ºC and 26ºC after 45 days demonstrated their pathogenicity, causing exudates and necrotic wounds on pears, similarly to cells from sterile water. This result reinforces our hypothesis of environmental waters as inoculum sources of this pathogen, as described for other *Erwinia* species [14,18].

In summary, our results demonstrate that *E. amylovora* can survive and remain pathogenic in natural waters, being its survival influenced, at least, by nutrient limitation, water temperature and bacterial competition. Low temperature appears to enhance *E. amylovora* persistence in water probably by slowing down the effect of starvation and water microbiota. Although to date the waterborne transmission of *E. amylovora* had been underestimated, the present results demonstrate that this risk exists, raising new questions on the role of water in fire blight epidemiology and control.

Acknowledgements This study was funded by projects AGL2004-07799-C03-02 and AGL2005-06982 from the Ministerio de Educación y Ciencia (MEC) of Spain. R.D. Santander and B. Águila thank MEC for their collaboration grants. M. Ordax also thanks MEC for a predoctoral fellowship. Dr. E. Marco-Noales wish to thank MEC (Programa INIA/CC.AA) and Agroalimed foundation from Generalitat Valenciana for her contracts. Authors are grateful to J. Catalá and J. Carmona for their help in some of the assays.

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The addition of copper sulphate to a non-selective medium improves the recovery of plant associated bacteria: *Erwinia amylovora* as a model

M. Ordax[1], **E.G. Biosca**[2], **M.M. López**[1], and **E. Marco-Noales**[1,*]

[1] Instituto Valenciano de Investigaciones Agrarias (IVIA), Departamento de Protección Vegetal y Biotecnología, Carretera Moncada – Náquera, km 4.5, 46113, Moncada,Valencia, Spain.
[2] Universidad de Valencia, Departamento de Microbiología y Ecología, Avenida Dr. Moliner 50, 46100, Burjassot, Valencia, Spain.

[*]Corresponding author: e-mail: ester.marco@ivia.es, Phone: +34 963424000-ext:70157

Copper is an essential micronutrient that at relatively high concentrations can increase bacterial growth on rich-nutrient media. In some bacteria copper also increases the exopolysaccharide production, like in *Erwinia amylovora,* causal agent of fire blight, a very serious and widespread disease of pome fruits and ornamental plants. The isolation and further identification of *E. amylovora* is the conclusive test for its presence in a vegetal sample. However, bacterial growth on solid media can be hampered by several factors. In oreder to optimize the recovery process we have modified a common nonselective medium, the King´s B (KB), by adding copper. In the copper-modified KB medium, *E. amylovora* colonies were early and easily differentiated by a yellow colour and high mucoid. The new medium was tested *in vitro* and *in vivo*, showing a recovery efficiency of *E. amylovora* higher than on the other media routinely employed, and resulting a useful tool to isolate this pathogen from the plant material.

Keywords plant epiphytic bacteria; fire blight; recovery; culture medium; King´s B; copper; exopolysaccharides.

1. Introduction

Copper ions are an essential micronutrient for all living organisms, but above certain levels they become toxic. However, relatively high copper concentrations can increase bacterial growth in nutrient-rich growth media [1] since most of copper ions become complexed to common media components, which reduces their toxicity [2, 3]. In addition, in some plant associated bacteria like *E. amylovora*, copper increases the production of exopolysaccharides (EPSs) [4].

E. amylovora is the causal agent of fire blight, the most serious disease of several pome fruits and ornamental plants in the *Rosaceae* family, reported in more than 40 countries. Its control is still not fully successful, representing a threat to the pome fruit industry. Consequently, to prevent the dissemination of *E. amylovora* especially to those countries free of the disease, there is a specific legislation in the European Union, where this pathogen is considered as a quarantine organism [5, 6]. One of the phytosanitary measures indicated is the use of suitable detection tools to avoid a further spread of the disease [7]. The standard PM7/20 reported by the European and Mediterranean Plant Protection Organization (EPPO) [8] recommends the isolation of *E. amylovora* (followed by a further identification) for a definitive confirmation of the pathogen presence in a vegetal sample.

The isolation of phytopathogenic bacteria such as *E. amylovora* from symptomatic plant material is relatively easy because the usually high number of culturable cells [8], but under adverse conditions it can be very low [9]. In fact, several plant bacteria adopt the viable but non-culturable (VBNC) state [10], remaining viable, but undetected on solid medium. Specifically, *E. amylovora* enter into this state by the presence of free copper ions [11] as well as under starvation conditions [12]. Therefore, the optimization of culture media to improve the recovery of this pathogen results necessary for both diagnostic and control purposes. Three media are advised for the maximum recovery of *E. amylovora* from plant material [8]: the non-selective media King´s B (KB) [13] and Sucrose Nutrient Agar (SNA) [14], and the semi-selective medium CCT [15]. Other culture medium used sometimes for *E. amylovora* isolation is MM_2Cu [4], based on the copper ability to increase the EPS levels. Taking advantage of the beneficial effects of copper on *E. amylovora*, in this work we have modified the KB medium by adding a selected copper sulphate concentration. The copper-modified KB medium was tested *in vitro* and *in vivo* for the isolation of the pathogen in the presence of the most frequent plant epiphytic bacteria in its host plants, using KB, SNA, CCT and MM_2Cu for comparative purposes.

2. Materials and Methods

2.1 Bacterial strains

Three strains of *E. amylovora* were initially tested on the KBCu medium: CFBP1430 (French reference strain), IVIA1892-1(Spanish strain from collection of Instituto Valenciano de Investigaciones Agrarias, IVIA), and Ea1/79 (German strain kindly provided by K. Geider). Moreover, epiphytic bacteria frequently found in its host plants were also assayed: *Pantoea agglomerans* (previously *Erwinia herbicola*) strain EPS411, and *Pseudomonas fluorescens* strain EPS347 (both from *Pyrus communis* in Spain and generously provided by E. Montesinos). Three levan (one of the EPS of *E. amylovora*) deficient strains (Ea1/79-18M, Ea1/79-5M, Ea7/74-LS7, kindly provided by K. Geider) were also tested.

2.2 *E. amylovora* growth in KB medium with sub-MIC copper concentrations

The MIC of copper for *E. amylovora* in KB broth was determined by adding copper as $CuSO_4$ (Sigma-Aldrich Chemie, Germany) at increases from 0.5 to 5 mM. Afterwards, to select the copper concentration for being added to KB medium, 20 µl from 48 h old cultures of *E. amylovora*, *P. agglomerans* or *P. fluorescens* strains in KB broth were separately inoculated with 10^5 cfu/ml in 200 µl of the same medium at increasing sub-MIC copper concentrations from 0.25 mM $CuSO_4$. The experiments were quadrupled and repeated independently using the Bioscreen C system (Labsystems, Finland), at 26º C with temporary shaking intervals of 1 h. The O.D. at 600 nm was recorded along 120 h.

2.3 Growth media and incubation conditions

The copper-modified KB medium was prepared by adding filter sterilized $CuSO_4$ (from a stock in distilled water, stored at 5ºC in dark) to sterile KB medium at 1.5 mM $CuSO_4$ as final concentration. For comparative purposes, the advised media by EPPO [8] (KB, SNA, CCT), as well as MM_2Cu [4] were used. Media were incubated at 26ºC during 48 h for KB [13], SNA [14] and the new one; 72 h, CCT [15]; and 96 h, MM2Cu [4].

2.4 Evaluation of KBCu medium *in vitro*

E. amylovora, *P. agglomerans* or *P. fluorescens* strains from KB broth were mixed in sterile distilled water at the final ratios 1:1, 1:10 and 1:100 for *E. amylovora* to the two epiphytic bacteria (each one separately or the two together for each ratio) at 10^8 cfu/ml. Afterwards, these suspensions were ten-fold serially diluted in phosphate buffer and plated, in duplicate, on the five growth media assayed. The culturable cells were enumerated after incubation. The experiment was performed independently twice.

2.5 Evaluation of KBCu medium *in vivo*

Pear trees (*P. communis* cv. Conference and cv. Blanquilla) and *Crataegus azarolus* from Spain (Logroño, La Rioja) that showed different fire blight symptoms were sampled and processed according to EPPO standard [8]. All the samples were plated on KB, SNA and CCT media, as well as on copper-modified KB and MM_2Cu. Colonial morphotypes appearing on each medium were registered, and those which showed an *E. amylovora* like-morphology were tested by ELISA-DASI [16] and PCR [17].

2.6 Statistical analysis

Statistical significance of the differences among the means was determined by a three-way factorial ANOVA analysis for *in vitro* (experiment, *E. amylovora* ratio and culture medium), and *in vivo* assays (experiment, type of plant material and culture medium). A p value < 0.05 was considered significant.

3. Results

3.1 Selection of copper sulphate concentration for adding to KB medium

The MIC of copper for *E. amylovora* in KB broth was 2.5 mM $CuSO_4$. Representative growth curves of *E. amylovora* and epiphytic bacterial strains in KB broth with sub-MIC $CuSO_4$ concentrations are shown in Fig. 1. Since results obtained with the three *E. amylovora* strains were similar, only the strain CFBP1430 is

represented. At concentrations below 1.5 mM $CuSO_4$ (Fig. 1A, B), growth of the epiphytic bacteria was significantly higher than that of *E. amylovora*, while at 1.5 mM $CuSO_4$ (Fig. 1C) the growth of epiphytic bacteria decreased down to the growth level of the pathogen (O.D. 600 around 0.8). Since above 1.5 mM $CuSO_4$ the *E. amylovora* growth became hampered (data not shown), 1.5 mM $CuSO_4$ was the concentration selected to be added to KB medium.

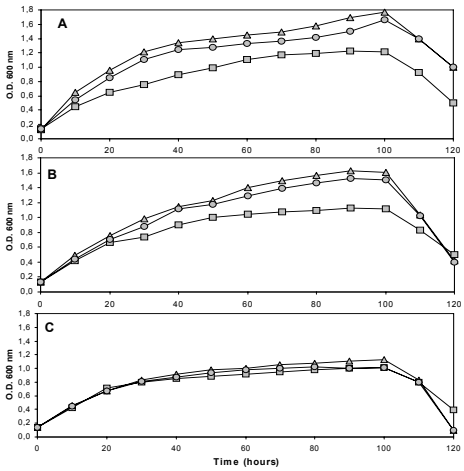

Fig. 1 Growth curves of *E. amylovora* CFBP 1430 strain (squares), *P. agglomerans* EPS 411 strain (triangles), and *P. fluorescens* EPS347 strain (circles), over 120 hours in KB broth supplemented with copper: A) 0.5 mM $CuSO_4$; B) 1 mM $CuSO_4$; C) 1.5 mM $CuSO_4$. Curves were obtained using the Bioscreen C system (Labsystems, Finland), at 26º C and with temporary shaking at intervals of 1 h.

3.2 Colonial morphology of *E. amylovora* on copper-modified KB medium

The colonies of *E. amylovora* strains assayed on the new medium (including the levan mutants) appeared yellowish, circular, domed, smooth, very mucoid, and with 2.5 - 4.5 mm diameter after 24-36 h incubation, while on KB medium they were creamy white, circular and smaller (1-1.2 mm) after 48 h [13]. A selection of 20 strains more of *E. amylovora* from different countries and hosts was also tested showing the same colonial morphology (data not shown). The colonies of the plant epiphytic bacteria assayed also showed a change in their colour, turning from deep yellow to orange in *P. agglomerans*, and from light to deep cream in *P. fluorescens*, so that they were easily distinguished from *E. amylovora*.

3.3 Plating efficiency of *E. amylovora* on KBCu medium *in vitro*

E. amylovora recovery from mixed cultures with *P. agglomerans* and/or *P. fluorescens* where the pathogen was in a lowest proportion (1:100) is shown in Fig. 2. The results with the three assayed strains of *E. amylovora* were very similar, so only CFBP1430 strain is represented. The culturability of the pathogen on KB medium with copper was significantly higher than on the other media, even when the pathogen was in the lowest proportion (Fig. 2). On the other hand, the two epiphytic strains assayed grew at significantly lower levels on new medium than on the other media assayed (data not shown).

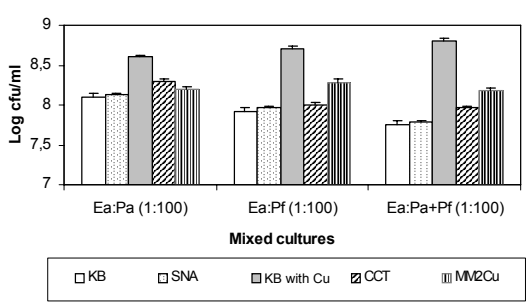

Fig. 2 Culturability of *E. amylovora* (Ea) (CFBP1430 strain) on KB, SNA and KB with copper (Cu) (after 48h), CCT (72h), and MM_2Cu plates (96h) at 26ºC from mixed cultures in ratio 1:100 with *P. agglomerans* (Pa) (EPS411 strain) and/or *P. fluorescens* (Pf) (EPS347 strain).

3.4 Plating efficiency of *E. amylovora* on KBCu medium *in vivo*

E. amylovora recovery from field symptomatic samples, like from shoot and branch, was significantly higher on KB with copper (99%) than on the other growth media (80-90%), or similar like in bark cankers (Fig. 3). All *E. amylovora*-like colonies were confirmed by ELISA-DASI [16] and PCR [17]. Likewise *in vitro* conditions, *E. amylovora* colonies on KB medium with copper were very mucoid, yellow, and easily distinguishable from those of epiphytic bacteria. In addition, the number of colonial morphotypes on new medium was lower than on the nonselective media KB and SNA (data not shown).

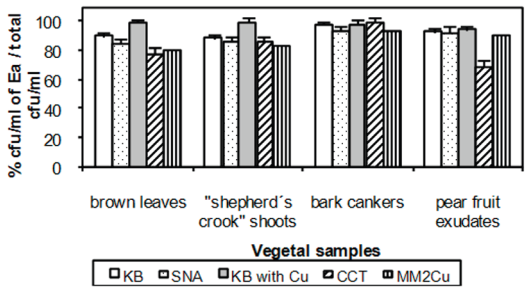

Fig. 3 Percentage of *E. amylovora* culturable cells regarding the total ones recovered from symptomatic samples (*C. azarolus*, and *P. communis* cv. Conference and Blanquilla). The isolation protocol followed was that stated in the EPPO standard PM7/20 [8]. Culture media and growth conditions were KB, SNA and KB with copper (Cu) (48h), CCT (72h), and MM2Cu plates (96h) at 26ºC.

4. Discussion

In this work, we have modified the most common nonselective growth medium used for the isolation of *E. amylovora*, the KB medium, by adding copper sulphate, to improve the recovery efficiency of this phytopathogenic bacterium from plant material.

On KB medium with copper, *E. amylovora* colonies showed a characteristic yellow colour and a high mucoid, due to the production of pigments [18] and an increase in the EPS levels [4] by copper. The *P. agglomerans* and *P. fluorescens* epiphytic strains assayed, as well as the plant native bacteria present in symptomatic samples, also acquired a more intense coloration on copper-modified KB plates, but clearly distinguishable from *E. amylovora*. Moreover, the typical colonial morphology of *E. amylovora* is visible earlier on KB with copper than on the other media assayed, due to the increase of EPS production by copper. Therefore, the copper addition to KB medium provides a useful differential trait for *E. amylovora* colonies, making easier their discrimination than on KB. Furthermore, in both *in vitro* and *in vivo* assays, the recovery efficiency of *E. amylovora* in the presence of plant epiphytic bacteria was significantly higher on KB with copper than on the other culture media employed for its isolation, and the epiphytic growth did not mask the presence of the pathogen. The comparison of copper-modified KB medium with the other media has shown several advantages of this new medium for the isolation and presumptive identification of the pathogen. For instance, *E. amylovora* colonies on KB and CCT can be confused with those of pseudomonads, requiring the monitoring of the plates under UV light [13, 15], while on KB with copper this is unnecessary. On the other hand, levan-deficient strains of *E. amylovora* that can be isolated from fire blight symptomatic samples can become unnoticed on SNA and CCT media [19]. However, as sucrose is not a component of KB with copper, these mutants showed on it the same morphology as levan producers strains, allowing their presumptive identification as *E. amylovora*. In addition, the new medium is composed by low cost components, and its preparation is easy and rapid.

Overall, we propose the inclusion of the copper-modified KB medium in the routine processing of plant samples for the isolation of *E. amylovora*. Due to the presence of copper, not only the differentiation of the pathogen colonies from those of other plant epiphytic bacteria results easier and faster than in other culture media frequently used up to now, but also the epiphytic growth levels are reduced, turning this medium into a useful tool for *E. amylovora* recovery from plant material.

Acknowledgements Work supported through projects AGL2004-07799-C03-02 and AGL2005-06982 from the Ministerio de Educación y Ciencia (MEC) of Spain, and GV-05/214 from Generalitat Valenciana (GV). M. Ordax thanks MEC for a predoctoral fellowship. Dr. E. Marco-Noales thanks MEC (Programa INIA/CC.AA) and Agroalimed foundation from GV for her contract. The authors specially thank Dr. J.M Quesada and M. T. Gorris (Centro Protección Vegetal y Biotecnología, IVIA) for PCR and ELISA analysis, respectively. We also wish to thank Dr. K. Geider (Max-Planck-Institut für Zellbiologie, BBA Institut, Dossenheim, Germany) and Dr. E. Montesinos (Universidad de Gerona, Spain) for the bacterial strains provided, as well as S. Rubio (Servicio de Investigación y Desarrollo Tecnológico, CIDA, Logroño, La Rioja) and M. Marín (Laboratorio Regional Finca "La Grajera", Logroño, La Rioja) for permitting us the sampling from orchards with fire blight symptoms.

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The effects of various organic wastes applied into eroded soil on dehydrogenase enzyme activity

Tugrul Yakupoglu[*,1], **Suheyda F. Hepsen**[1], **Nutullah Ozdemir**[1] and **Ridvan Kizilkaya**[1]

[1] Ondokuz Mayis University, Faculty of Agriculture, Department of Soil Science, 55139, Samsun, Turkey

[*]Corresponding author: e-mail: tugruly@omu.edu.tr; phone: 00903623121919; fax: 00903624576034

It is known that, organic wastes added to soil improve many soil properties. Bio-solid (BIO), tea production wastes (TEW) and tobacco production wastes (TOW) are some organic wastes used for this target. The objective of our study was to find out the effects of these organic matter sources on dehydrogenase enzyme activity (DHA) in different levels of eroded soils. To determine different soil erosion levels (slightly, moderately and severely), erosion ratio (ER) and structural stability index (SSI) parameters were used. ER values of slightly, moderately and severely eroded soils were determined as 5.36, 6.65 and 12.17 while their SSI values were determined 78.3, 77.8, and 68.7 respectively. Vertic calciudoll samples used in this research were taken from surface (0 to 20 cm depth) located on agricultural areas of Samsun, Northern Turkey. This area has been used as agricultural activity for a long time. This study was conducted by applying four different doses of BIO, TEW and TOW (0, 2, 4 and 6 %, basis dry weight) into eroded soils under greenhouse condition. Each treatment was replicated three times in a split block design. After eighteen weeks incubation period, DHA was determined in all pots. According to analysis results, relation between erosion level and DHA were found as negative. TEW and TOW were increased DHA values in all erosion levels contrast to itself control application. The effects of different organic wastes on DHA were increasing depended on application dose. Additionally, BIO application was increased DHA with 2 % doses whereas it was decreasing DHA with 4 % and 6 % doses. These results were found statistically significant at $P<0.001$.

Keywords dehydrogenase; erosion; organic waste; soil

1. Introduction

Recently, waste materials depending on increased industry and urbanisation result in problems for environment due to difficulties in their store. This serious condition is occurred that new alternative solves ought to found. Concerns about environmental quality have led to the introduction of alternative disposal methods such as the use as nutrient source to plants and as soil conditioners (1). In a research, some researchers reported that the use of industrial organic matter in agricultural lands can be justified by the need of finding an appropriate destination for waste recycling (2, 3).

Especially organic wastes can used many ways in agricultural applications. Some of these materials are bio-solid (BIO), tea production waste (TEW) and tobacco production waste (TOW). Generally, when these organic wastes are mixed into problematic soils then their some physico-chemical and biological properties can improve (4, 5, 6). In addition, many researchers (7, 8, 9) showed that organic waste application in soil was decreased erodibility, and therefore soil may be also increased soil productivity. It is well known that soil microorganisms and their activities are a significant component of soil quality and microorganisms play vital roles in soil fertility and primary production through organic matter decomposition and nutrient cycling. Thus, it is meaningful to evaluate the soil biological aspects of productivity within the context of overall system function. Dehydrogenase activity is widely used in evaluating the metabolic activity of soil microorganisms. Since dehydrogenases are not active independently of the parent microbial cell as extracellular enzymes in soil, measurement of the dehydrogenase is a good overall indicator of microbial activity and oxidation of organic matter (10, 11).

The aim of this study was to determine the effects of organic wastes such as bio-solid (BIO), tea production waste (TEW) and tobacco production waste (TOW) mixed into different levels of eroded soils on dehydrogenase activity.

2. Materials and Methods

2.1. Soil sampling

In this study, soil samples (0-20cm depth) were taken from different levels of eroded area in Samsun-Turkey. This site is located in the Black Sea Region, Northern Anatolia (Latitude 41º 19'N; Longitude 36º 02'W) as shown Figure 1. The climates is semi humid (R_f= 47.21) with monthly mean temperatures ranging from 6.6ºC in February to 23ºC in August. The annual mean temperature is 14.2 ºC, annual mean precipitation 670 mm (12).

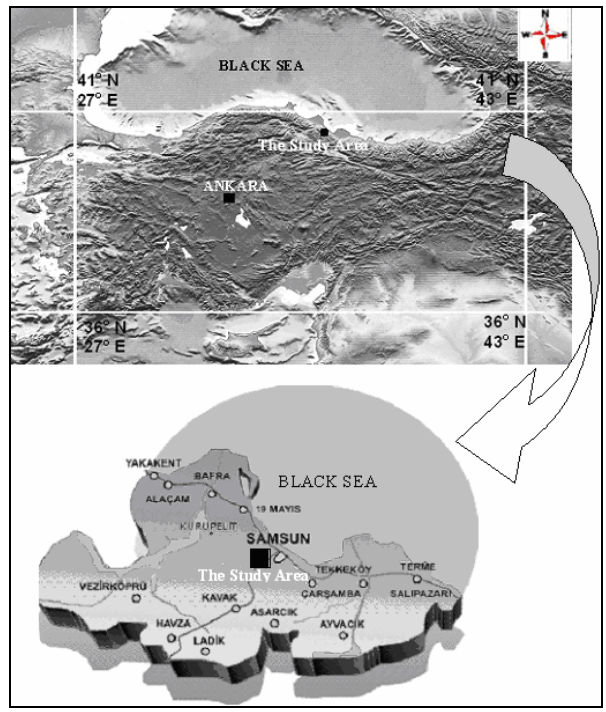

Fig. 1 Location map for soil sampling area

2.2. Soil physico-chemical properties

Soil physical and chemical properties were determined by means of appropriate methods: particle size distribution (texture) by hydrometer method, pH and electrical conductivity (EC) in 1:2.5 (w/v) in soil:water suspension by pH-meter and EC-meter, cation exchange capacity (CEC) by Bower method, organic matter content by a modified Walkley-Black method, $CaCO_3$ content by Scheibler calsimeter, field capacity by pressure plate (13, 14).

Erosion ratio (ER) and structural stability index were determined according to references 15 and 16.

2.3. Organic wastes

Tea and tobacco plants are commonly grown in the Eastern and Middle Black Sea Region of Turkey. Therefore, there is much tea (TEW) and tobacco (TOW) production waste in this region. These organic wastes were sampled from tea and tobacco production regions. Bio-solid (BIO) was obtained from the Bafra Wastewater Treatment Plants, Samsun, Turkey. BIO was anaerobically digested with a mixture of primary and waste activated sludge typically entering the digester. All organic wastes were dried and sieved to less than 0.50 mm. The properties of the organic wastes were expressed on a moist-free basis and analyzed by standard procedures, given in reference 17.

2.4. Experimental design

Soil samples were dried in a laboratory, not in an oven, to workable moisture content and sieved through 4-mm screens. The soil samples (4000 g air-dried soil) were placed in 5 L cylindrical plastic pot. The soil samples were treated with the organic wastes (BIO, TEW and TOW) at four different levels (0, 2, 4 and 6 % w/w) and each treatment was replicated three times in a split block design. The untreated soil served as control. All of the pots were incubated for eighteen weeks under greenhouse condition. The soil moisture was kept at the field capacity by adding water at regular intervals throughout the incubation. At the end of the incubation period, dehydrogenase activity (DHA) of soil samples collected from the pots was determined. All DHA determinations were performed for the soil sample in triplicate, and all values were reported averages of the three determinations expressed on an oven-dried sample basis at 105 ^{0}C for 48 h.

2.5. Dehydrogenase activity

To 6 g of sample 30 mg glucose, 1 ml of 3% TTC (2,3,5-triphenyltetrazoliumchlorid) solution and 2.5 ml pure water were added and samples were incubated for 24 h at 37^{0}C. The formation of TPF (1,3,5 triphenylformazan) was determined spectrophotometrically at 485 nm and results were expressed as μg TPF g^{-1} dry sample (18).

2.6. Statistical analysis

All data were analyzed using SPSS 11.0 statistical software (SPSS Inc.). Analysis of variance (ANOVA) was carried out using split block design; where significant F-values were obtained, differences between individual means were tested using the LSD (Least Significant Difference) test, with a significance level of $P<0.01$.

3. Result and Discussion

3.1. Soils

Some soil physico-chemical properties and erosion indices determining results (ER and SSI) are presented in Table 1.

Table 1 Some physico-chemical properties of soils

		Soil number		
Soil properties		1	2	3
Particle size distribution, gkg^{-1}	Sand (S)	146	131	152
	Silt (Si)	260	308	317
	Clay (C)	594	561	531
Textural class		C	C	C
pH		8.0	8.1	8.1
Organic matter content (OM), %		0.99	0.84	0.83
EC_{25^oC}, dSm^{-1}		0.78	0.65	0.64
Cation exchange capacity (CEC), $meq100g^{-1}$		37.4	23.9	21.4
Lime content, %		16.6	19.4	21.9
Field capacity, %		38.3	35.9	33.1
ER		5.36	6.65	12.17
SSI		78.3	77.8	68.7
Erosion levels		Slightly	Moderately	Severely

Different levels of eroded soils were classified as Vertic calciudolls according to the U. S. Taxonomy (19). The soil physico-chemical properties (texture, pH, EC, organic matter, CEC and $CaCO_3$) were generally similar for all soil samples. However, Soil 3 had the lowest clay content than the other soils. Similarly, field capacity was the lowest in soil 3. For this reason, erosion indices determining may be calculated the different levels in soils. This means, soil 1, 2 and 3 can be slightly, moderately and severely eroded soil, respectively.

3.2. Composition of organic wastes

Among the organic wastes used in this study, TEW had the highest organic matter (92.72%) while that of BIO was the lowest (37.00%). Regarding N content, TEW again had the highest N content (2.46%) and the lowest N content belong to TOW (1.97%). C:N ratio of the organic wastes ranged from 9.3 to 21.8 and the highest level C:N ratio observed in TEW while that of lowest is BIO. In addition these organic wastes contained major nutrients such as P_2O_5, K_2O, CaO which are agronomically important (Table 2).

Table 2 Composition of organic wastes in measured variables

Organic waste	TEW	TOW	BIO
Organic matter, %	92.71	66.21	37.00
N, %	2.46	1.97	2.40
C:N	21.8	19.5	9.3
P_2O_5, %	0.48	0.45	2.98
K_2O, %	5.83	4.71	0.28
CaO, %	1.05	3.91	11.50

3.3. Dehydrogenase activity

The changes of DHA in organic wastes amended soils at the end of the incubation period are shown in Figure 2. Considerable variations in DHA were found for the different doses of organic wastes at the end of the incubation period in different levels eroded soils. Statistically significant variations were found in DHA at organic waste types and waste doses. DHA were also affected by soil erodibility. The analysis of variance of the results obtained in our experiment showed that all factors (organic waste types, waste doses and erodibility levels) significantly influenced DHA (Table 3). At the end of the incubation, the DHA measured in treated soils were statistically different from those measured in the control soils.

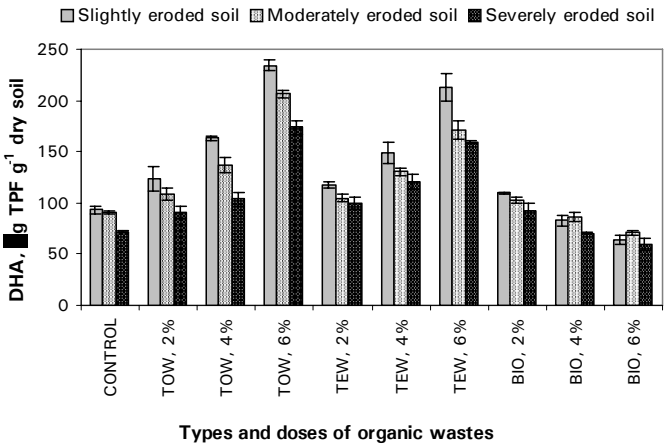

Fig. 2 Changes in the DHA the soils amended with different types and doses of organic waste. Bars represent the standard deviation of mean values

In this study, results showed that the relationships between erosion level and DHA were found as negative. The higher DHA were observed in slightly eroded soil than the other soils at all organic waste types and doses. TEW and TOW were increased DHA levels in all soils compared to control. The effects of different organic wastes on DHA increased depending on application dose. Figure 2 shows that the DHA levels of the soils and 6% doses of TEW and TOW application were higher than those of 2 and 4% application doses and control. Additionally, BIO application increased DHA with 2 % doses whereas; it decreased DHA with 4 % and 6 % doses.

Soil DHA reflects the total range of oxidative activity of soil microflora and, consequently it may be a good indicator of microbiological activity in the soil (20). DHA increased in TOW and TEW amended soils with respect to the control soil (Fig.2). Several studies (21, 22) showed several organic waste treated soil higher DHA than untreated soil. Treated organic waste might have decomposed, resulting in higher enzymes in soil. In addition, increased content of organic carbon and nutrients may be caused increasing DHA in soil. It is possible that the organic waste treatment stimulated microbial production of enzymes in soil. However, DHA decreased in BIO amended soils compared to the control. Although BIOs had a high level organic matter and nutrients, they also contained potentially toxic metals (e.g., Cd, Zn, Cr). DHA was probably negatively affected by heavy metal contents of soil. Numerous studies (23, 24) have demonstrated negatively effects of heavy metals on DHA. In our study, inhibition of DHA was probably due to several metals and not solely to one metal component.

Table 3 Statistical analysis results for DHA values

	A	**B**	**AxB**	**C**	**AxC**	**BxC**	**AxBxC**
F-value	251.437***	857.212***	24.923***	636.041***	7.249***	345.778***	6.347***
$LSD_{0.01}$	3.485	3.485	6.036	4.024	18.638	6.969	12.071

A= soils, B= types of organic waste, C= doses of organic waste; *** $P<0.001$

4. Conclusion

We assume that the TOW and TEW have stimulating effects on DHA in different levels of eroded soils, due to the quantity and quality of the organic waste incorporated into soil, and the microbial growth caused by the addition of organic compounds to the soil. In general, the application of TEW and TOW caused the most beneficial effects on DHA in soils among the investigated types of organic wastes on different levels of eroded soil. The use of these organic wastes can contribute to an enhancement of the level of organic matter and the fertility of the agricultural soils. Furthermore, organic wastes had a stronger impact on DHA in soils as compared with control. However, many risk assessments and regulations for the application of BIO are based on total and available metal concentrations in soil. DHA in BIO amended soils especially 4 and 6% application doses also decreased dramatically at all soil types. Hence, further studies are needed to explore the relevancy in using DHA to reflect different types of BIO and soil.

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Use of vinasses in the control of fungi phytopathogens

Santos M.*· Diánez F.· de Cara M.· Tello J.C.

Departamento de Producción Vegetal. Universidad de Almería. La Cañada de San Urbano. Almería. 04120

* E-mail: msantos@ual.es

The purpose of this research was to study the biocide effect of three agroindustrial subproducts, concretely sugar beet, sugar cane and wine vinasse. Two tests were carried out. The first centred on studying the action of the three agroindustrial subproducts *in vitro.* In dilutions at initial doses of 1%, 3% and 5%, their performance against six phytopathogenic fungi was analyzed: *Fusarium oxysporum* f.sp. *melonis* race 0, *Fusarium oxysporum* f.sp. *melonis* race 1, *Fusarium oxysporum* f.sp. *radicis-cucumerinum, Sclerotinia sclerotiorum* (as representatives of the Mycetae or Fungi kingdom, whose cell walls contain chitin) and *Pythium aphanidermatum* and *Phytophthora parasitica* (as representatives of the Chromista kingdom, whose cell walls contain cellulose). Next, the antagonistic capacity of the solutions assayed *in vitro* was tested in soil, studying the incidence of the subproducts on the Fusarium populations in these soils.

Results from *in vitro* testing determined that wine vinasse is what shows a 100% capacity to suppress fungal growth with concentrations that are not very high, between 5% and 7% for *Fusarium oxysporum* f.sp. *melonis* race 0, *Fusarium oxysporum* f.sp. *melonis* race 1, *S. sclerotiorum*, *P. aphanidermatum* and *P. parasitica* and 10-15% for *Fusarium oxysporum* f.sp. *radicis-cucumerinum.* On the other hand, sugar cane vinasse produced an increase at high concentrations and sugar beet vinasse showed an approximate 100% suppressor effect on fungal growth for only some of the phytopathogens tested: *S. sclerotiorum* (15%), *P. aphanidermatum* (7%), *P. parasitica* (15%) and *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (15%).

In the soil samples analyzed none of the three vinasse extracts decreased fusaric microbiota, producing an increase in the three samples tested. This would implicitly convey an improvement in soil quality by producing a potential increase in bacterial and fungal microbiota.

Therefore the continuity of this research is necessary, carrying out field experiments so potential lower concentrations can be determined that generate disease suppression in more complex systems.

Keywords agroindustrial subproducts, vinasses, phytopathogens, biological control.

Introduction

One of the greatest challenges of the past several years that researchers and agricultural technicians are now facing is to find alternatives to methyl bromide (MB) and pesticides to control plant pests and diseases in general, due to their negative effects on the environment and public health. On the other hand, the increase of residue production in modern society is an unavoidable fact. Therefore, current predictable socioeconomic conditions require quite different residue management than in past decades. Various alternatives to palliate the effects of these residues concern several population levels since solely by ascribing a value to them would they stop being considered residues, and perhaps be accepted within the concept of resource or by-product.

One might therefore emphasize the need and importance of research on the biocidal effect of agroindustrial and stockbreeding subproducts to serve as a foundation for a registry of a series of biopesticides. This would be highly useful in agriculture while simultaneously allowing for compliance to international commitments on environment and health, such as the Montreal Protocol in the case of methyl bromide and the Stockholm Treaty on persistent organic pollutants [2].

Previous research has revealed the efficacy of biofumigation in the control of phytopathogenic fungi and nematodes. It has confirmed that various subproducts used for this end possess an efficacy similar to that of conventional fumigants by increasing populations of saprophagous organisms and the biomass of plants grown on biofumigated soils. However, their efficacy depends on the dose and application protocols. For these reasons the optimization of field application techniques are necessary so that these subproducts can be used as an alternative for controlling plant pathogens, as well as to evaluate to what point they work as organic enhancers by boosting physical and chemical soil properties. Thus, crop profitability would be increased, preventing the environmental impact of inadequately managed agroindustrial and stockbreeding subproducts.

Therefore, developing procedures for using and transforming agroindustrial subproducts could achieve the deployment of alternative, environment-friendly methods for crop protection, compatible with the demands of ecological agriculture.

This research evaluates the biocidal effect of three agroindustrial subproducts: sugar beet, sugar cane and wine vinasse. It centres mainly on two aspects, on the one hand the action of the three above-mentioned

agroindustrial subproducts were studied *in vitro*. Their performance was analyzed in solutions at initial doses of 1%, 3%, and 5% against six phytopathogenic fungi: *Fusarium oxysporum* f.sp. *melonis* race 0, *Fusarium oxysporum* f.sp. *melonis* race 1, *Fusarium oxysporum* f.sp. radicis-cucumerinum, *Pythium aphanidermatum*, *Phytophthora parasitica* and *Sclerotinia sclerotiorum.* According to the results obtained, the establishment of optimum doses to control these fungi was sought. On the other hand, the incidence of the above-mentioned subproducts was studied on Fusarium populations in several soils.

Materials and methods

Evaluation of fungal growth inhibition by vinasse

For growing the various fungal species, isolates were maintained in PDA medium in 9 cm diameter Petri dishes. Fungi were maintained by weekly inoculation for *Phytophthora parasitica, Fusarium oxysporum* f.sp. *melonis* race 0, *Fusarium oxysporum* f.sp. *melonis* race 1, *Fusarium oxysporum* f.sp. *radicis-cucumerinum*, and approximately every four days for *Pythium aphanidermatum* and *Sclerotinia sclerotiorum.*

Inoculants for the assays were obtained from maintenance dishes, 5 mm diameter discs, and removed from the periphery of approximately 6 cm diameter colonies in active growth with the help of a punch. Therefore, the inoculant is basically composed of edges of mycelia in growth and not of conidia and other types of latent propagate that require a period of incubation before germination.

To evaluate the suppressor effect of vinasse on fungal growth, tests were carried out *in vitro* with dilutions of sugar beet vinasse, sugar cane vinasse and wine vinasse in PDA at 1%, 3%, and 5% (and later at 7%, 10% and 15%). The phytopathogenic fungi used in the assays were obtained from the maintenance dishes. With the help of a punch, 5 mm discs were taken, always from the periphery of the colonies and in active growth. Each disc was placed in the centre of a Petri dish. The dishes were incubated in the oven in darkness at 25-26ºC for one week with the exception of *Pythium aphanidermatum* and *Sclerotinia sclerotiorum* which were incubated for 3 days. Five repetitions were carried out and the results were compared to controls with no vinasse application. Growth was analysed by measuring the diameter of the centre of the vine. Two measurements were taken, i.e. two perpendicular diameters. In the end the average of both values was used as representative data. Lastly the results were statistically analyzed with the STATGRAPHICS 5.1 (S.G.S., 2001) program to evaluate the effect of extract concentrations on the efficacy of fungal suppression.

Study of the antagonistic capacity in soil of the solutions assayed *in vitro*

Fusaric microbiota from three soil samples coming from a bean crop originating in Asturias were studied and quantified. The effect of applying sugar beet, sugar cane and wine vinasse at 1%, 3%, 5%, 7%, 10%, and 15% on populations of Fusarium species in those samples was studied. Total fusaric microbiota was analysed before and after application of the subproduct [12].

This analysis was made by an in-dish count of the units forming *Fusarium* spp. colonies, expressed as $CFU \cdot g^{-1}$. The soil or substrate dilution technique was used directly on the soil-plate, as described by Warcup [13].
After being ground and weighed, the soil or substrate was incorporated into the culture medium in a state of fusion near the temperature of solidification. The culture medium used for the analyses was invented by Komada [6] and modified in the composition of the solution of antibiotics [12]. This medium was chosen because of its high selectivity for the *Fusarium* species and for its relative simplicity in differentiating some of them based on pigmentation, aspect and colony growth rate.

The analysis methodology complied with what Tello et al. described [12], based on the one elaborated by Rouxel and Bouhot [9]. For stabilization soil samples were dried at room temperature for a variable time period (between 3 to 7 days) according to humidity in the sample. Once samples were dry, an aliquot was taken and ground in a porcelain mortar and sieved through a 200 μm opening screen. For each sample to be analyzed, four glass flasks were prepared with 20 g of soil each, dried at room temperature, having a maximum particle size of 200 μm. Each flask was considered as one repetition and was weighed to a precision of 0.001 g before and after analysis. The difference determined the weight of the substrate in each repetition. A small quantity of soil which was homogenised with 10 ml of Komada culture medium (temperature of 40-42ºC) was added to each 9 cm diameter Petri dish with a micro-spatula. Four dishes were prepared for each glass flask or repetition, totalling 16 dishes for analysis. Petri dishes were incubated at room temperature for 5-10 days. To determine total fusaric microbiota, a count was made after the first four days, by ticking them off on the underside of the Petri dish. Three or four days later a final annotation was made.

The procedure for analyzing samples after application of the product is the same as previously described. The only difference lies in that once samples were ready, 10 ml of previously prepared dilutions of sterilized water

and vinasse at the corresponding concentrations were added to each one of them. After that addition soil samples were left to dry at room temperature as long as necessary, then proceeding to the described steps. After the count and characterization of the units forming *Fusarium* spp. colonies ($CFU \cdot g^{-1}$), a comparative analysis of total fusaric microbiota was established before and after applying the subproduct [12]. Lastly, a statistical analysis of the results was made to evaluate the effect of extract concentrations by using the STATGRAPHICS 5.1 (S.G.S., 2001) program.

Results

Evaluation of fungal growth inhibition using vinasse

The assay aimed to determine the *in vitro* inhibitor effect of vinasse waste on fungal development of six pathogens: *P. aphanidermatum, P. parasitica, F. oxysporum f.sp. melonis* (Fom), f.sp. *melonis* race 1 (Fom 1), *f.sp. radicis-cucumerinum* (FORC) and *S. sclerotiorum.* Initial doses were 1%, 3% and 5% with the intention of proceeding to lower dilutions and establishing optimum doses for controlling fungi. Since no significant visual differences were observed once the assay with these doses had finalized, doses were instead increased to 7%, 10% and 15%.

Figure 1 shows the percentage of inhibition from sugar beet vinasse against the various fungi tested. It was observed that sugar beet vinasse at more or less high concentrations obtained an inhibition of 100% only in the case of some fungi. Inhibition started at 7% in the case of *Pythium* while a concentration of 15% was necessary for *Phytophthora, Sclerotinia* and FORC. However 100% inhibition of Fom and Fom-1 was not found at any tested concentration.

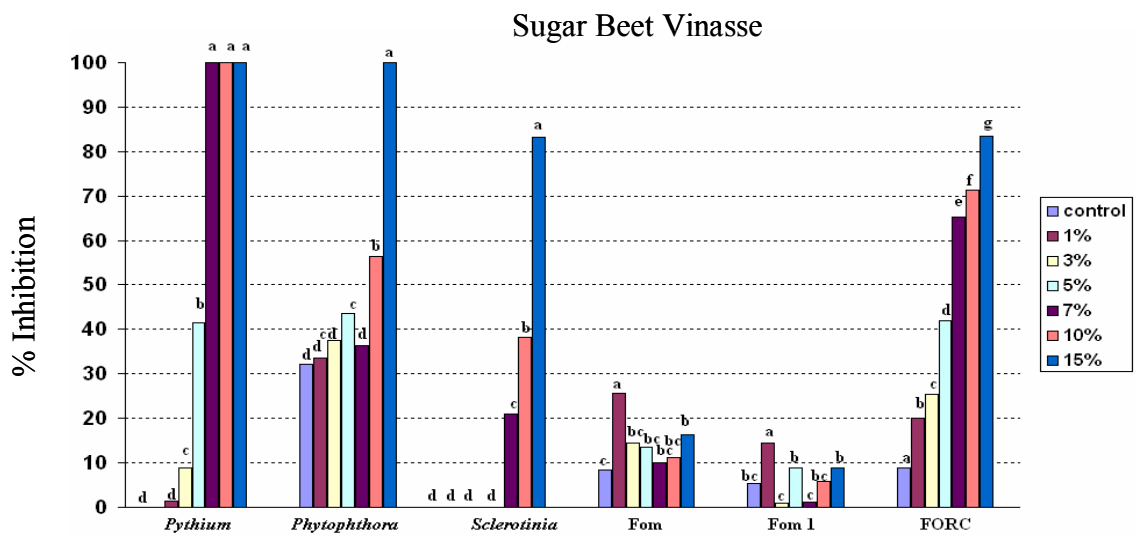

Figure 1. *In vitro* inhibitor effect (%) of Sugar Beet Vinasse waste on fungal development of six pathogens.

In spite of obtaining maximum inhibition at elevated concentrations, at lower concentrations significant differences already existed with regard to growth in the control: at 3% in the case of *Pythium* and *Phytophthora*, at 7% for *Sclerotinia* and even at 1% for FORC.

Figure 2 shows that sugar cane vinasse did not succeed in inhibiting total mycelia development for any of the phytopathogenic fungi tested. It did not even manage to demonstrate any effect on growth at all, as in the case of *Pythium* where there was maximum fungal growth was for all the concentrations tested.

Wine vinasse, as can be observed in Figure 3, is undoubtedly what obtained the bests inhibition results, attaining approximately 100% inhibition on all fungi tested. Starting at 5% in the case of *Pythium* and *Phytophthora*, inhibition already reached 100%. This was the minimum concentration to present significant differences compared to the control. For *Sclerotinia* and FORC, concentrations of 7% and 10% produced almost total inhibition while the 15% concentration obtained total inhibition of fungal growth. Best results for Fom and Fom-1 were obtained at 7%, with significant differences at a concentration of 3% compared to the control.

Table 1 summarizes concentrations of the various extracts that produced an approximate 100% suppression of growth for all six fungi. It was observed that the application of sugar cane vinasse did not achieve maximum inhibition of fungal growth for all the phytopathogenic fungi tested.

No bibliography exists for comparing results to other authors, since only scarce articles in literature make reference to the use of vinasse as fertilizers or improvers of physicochemical soil properties.

Work by Bello researched the action of agroindustrial and livestock subproducts in crop protection, studying their efficacy in controlling phytopathogenic nematodes. It confirmed that they possess an efficacy similar to conventional nematicides in the control of root-knot nematodes (*M. incognita*), by increasing the population of saprophagous nematodes. Thus, in the case of vinasse extracts a concentration of 0.2% is enough to obtain *M. incognita* suppression. Applications of vinasses at 2% alone or combined with manure and covered with plastic are also highly efficient in the control of *X. index*. A similar efficacy was observed in the application of vinasses without plastic but with organic matter, but was no longer effective after five months when used alone without plastic (A. Bello, personal comunication).

These results from wine vinasse extract seem to agree with results by Diánez et al. [3] for grape marc compost, where suppressiveness was detected against nine phytopathogenic fungi. Concretely in the *in vitro* evaluation of aerated teas from grape marc compost, high inhibition suppressiveness of mycelia growth was obtained (always over 80% under all tested conditions) when using filtered teas.

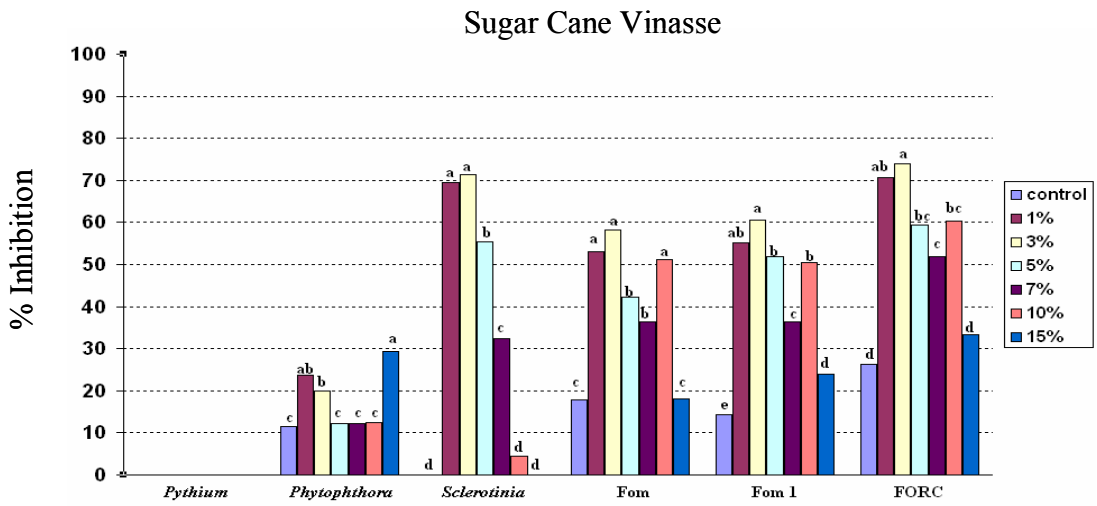

Figure 2. *In vitro* inhibitor effect (%) of Sugar Cane Vinasse on fungal development of six pathogens.

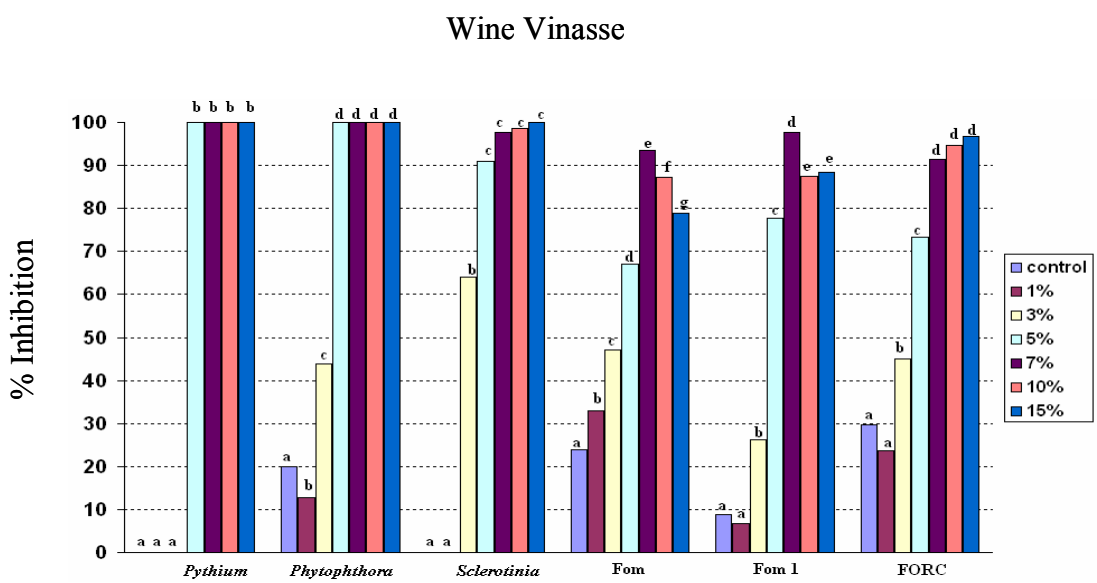

Figure 3. *In vitro* inhibitor effect (%) of vinasse waste on fungal development of six pathogens.

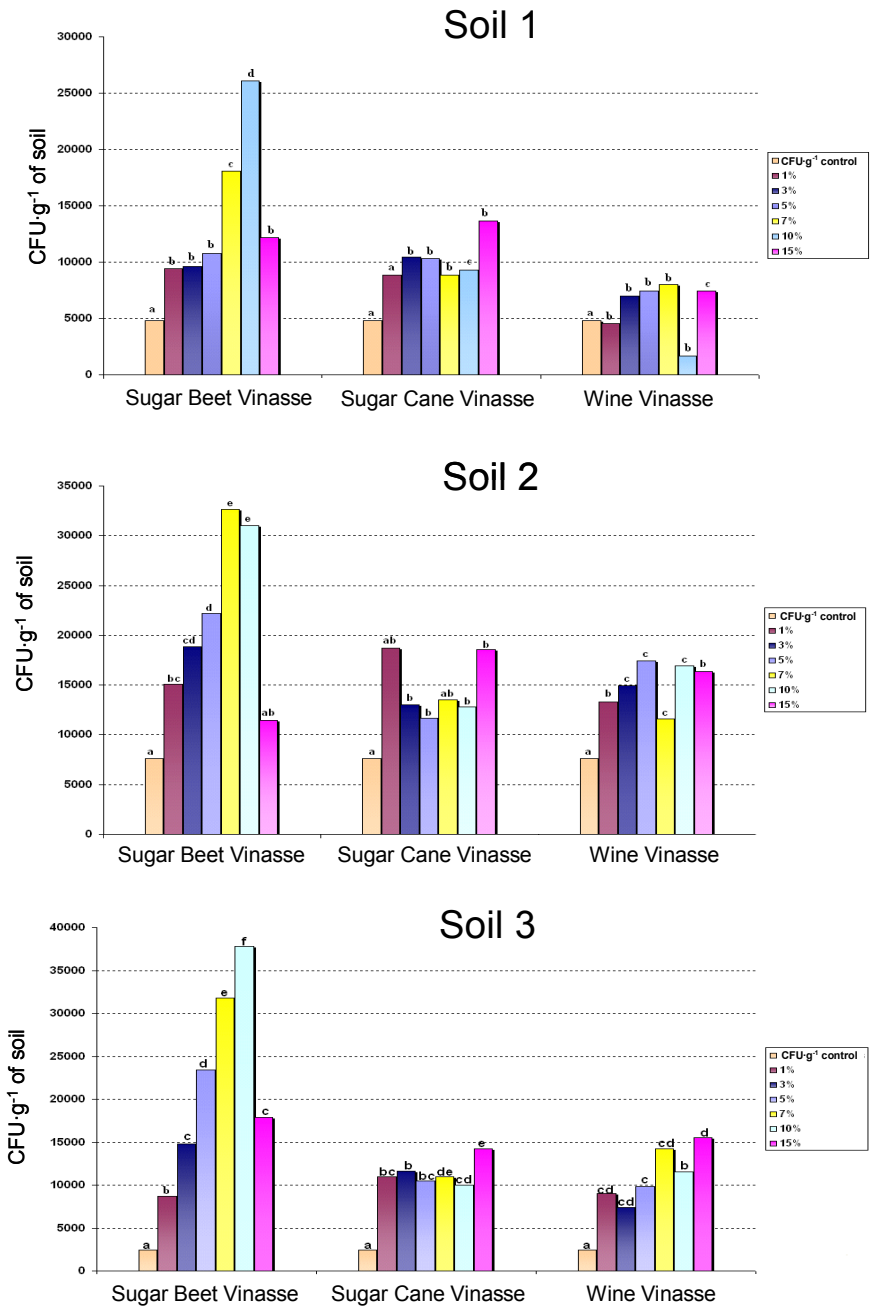

Figure 4. Effect of different concentrations and type of vinasse on populations of the fungus *Fusarium* in soil (1, 2 and 3)

Table 1. Vinasse concentration which caused completely inhibitory effect on mycelial growth.

	Sugar Beet Vinasse	Wine Vinasse	Sugar Cane Vinasse
P. aphanidermatum	7%	5%	-
P. parasitica	15%	5%	-
S. sclerotiorum	15%	5%	-
Fom	-	7%	-
Fom1	-	7%	-
FORC	15%	10-15%	-

Evaluation of the antagonist capacity on soils of solutions tested *in vitro*

Once the previous analysis was finished, fusaric microbiota from the three soil samples was studied and quantified. Soil had been taken from pastures transformed for agriculture in Asturias without any presence of pathogenesis. Diseases were not described in those soils and no variation in their populations existed either before or after the biosolarisation process. The antagonistic capacity of previously assayed dilutions *in vitro* was studied in comparison to the fusaric populations present in those samples. This analysis was carried out by an in-dish count of the units forming *Fusarium* spp. colonies, and expressed as $CFU \cdot g^{-1}$. Three graphs (Figure 4) are shown below for each soil sample relating the units that form *Fusarium* spp. colonies expressed as $CFU \cdot g^{-1}$. compared to the concentration for a 95% confidence level.

As can be observed, all three vinasse extracts give rise to an increase of fusaric microbiota in the analysed samples, producing a maximum increase of two to six times more in the case of soil samples No. 1 and 2, and up to 5 times more in the case of sample No. 3. It is difficult to contrast data from other authors due to the scarcity of articles in literature that only make reference as mentioned above to the activity of vinasse as a fertilizer or improver of physicochemical soil properties.

García and Rojas, [5] found that the soil-borne bacteria population incubated with vinasse grew rapidly from the first to the fourth week of incubation even with a dose as low as 150 $m^3 \cdot ha^{-1}$ of diluted vinasse (10% m/m concentration of solids), with a population increase of 25.3% compared to soil without vinasse. Readily usable sugars such as pentose are easily decomposed by soil microorganisms during the decomposition of organic matter, at the same time as nitrification and denitrification are altered, a situation that can be corrected through the addition of vinasse. For example, nitrogen fixation theoretically requires 1.7 g of carbon per gram of fixed nitrogen. So, if the soil is low in carbon content, the addition of vinasse can improve this process. That is, vinasse can be used to promote microbial activity in the decomposition of field residues. In addition, several researchers have attributed part of the responsibility for improving the physical structure of the soil to organic matter which increases its microbial population and activity and heightens water infiltration. They attribute these effects to products and secretions from microorganisms when decomposing organic matter, which unite soil particles [4].

The specific fact that wine vinasse achieves a suppression of fungal growth *in vitro* assays for *Fasarium oxysporum* f.sp. *melonis* race 0, *Fusarium oxysporum* f.sp. *melonis* race 1 and *Fusarium oxysporum* f.sp. *radicis-cucumerinum* and, as just seen, increases fusaric microbiota in soil samples is a difficult matter to explain and contrast due to limited bibliography. In the face of this fact, diversities between one case and another must be emphasized, since the conditions of each test are different and results from an *in vitro* analysis are not easy to correlate to a soil analysis. For, principally, a great diversity of microorganisms exist in the soil whose development can be furthered or thwarted by adding the extract during the existing incubation period by establishing antagonistic or symbiotic relationships. In addition, when Fom, Fom-1 and FORC are considered, they refer to phytopathogenic fungi. But soil analyses only analyze fusaric microbiota in general without reaching the species level. Therefore the possibility exists that the increase of initial fusaric microbiota from tested soil samples is due to an increase of saprophagous, but not phytopathogenic, *Fusarium* species. So, verifying results from plant inoculations would be adequate, observing whether vinasse extracts really generate an increase in pathogenesis. Organic by-products originating from industrial processes can represent an important source of nutrients, especially for organic fertilization [11]. Despite the nutrients contained in the vinasse, under dryland conditions this byproduct may negatively affect soil structure, nutrient uptake, and crop yield and quality [1, 10], although other studies have indicated that such negative effects are not observed with irrigated maize [11]. Some authors have suggested that the problems associated with fresh residues (such as BV) may be overcome by co-composting with solid agricultural wastes [7,8].

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Utilization of organic and mineral amendments to control potato bacterial wilt disease

H.M. Elhenawy[*, 1], N.Y. Abd El-Gafar[2], and F.G. Fawzi[3]

[1] Central Administration for Plant Quarantine (PQCA) & Potato Brown Rot Project (PBRP), Agriculture Ministry, Dokki, 12311 Giza, Egypt.
[2] Plant Disease Department, Faculty of Agriculture, Ain Shams University, P.O.Box. 68 Hadayek Shobra, 11241 Cairo, Egypt.
[3] Agriculture Research Center & Potato Brown Rot Project (PBRP), Dokki, 12311, Giza, Egypt

[*]Corresponding author: e-mail: Haidi_Elhenawy@yahoo.com. Phone: +20123241722

Potato brown rot; known as bacterial wilt disease, is a serious disease causing problems in the warm regions of the tropics and subtropics. It has been reported also in cool climates of North Western Europe. Primary infection by *Ralstonia solanacearum* bacteria occurs through roots or the stolons as soil born disease. This study carried out to study the effect of organic matters and mineral fertilizers as soil amendments on bacterium population of *Ralstonia solanacearum* and disease severity (virulent and avirulent forms), under artificial inoculation condition. Result indicated that bacterium population and disease severity were significantly reduced when treated with the soil amendments "Garlic" and "Potassium Sulfate" after 90 days in comparison to the control treatment. Additionally, it was clear from the experiment that organic matters were more successful in the virulent forms reduction.

Keywords Potato, Bacterial Wilt, *Ralstonia solanacearum*, Soil Amendment, Garlic, Cabbage, Camphor, Organic Matter, Mineral Fertilizers, Ammonium Nitrate, Potassium Sulphate, Ammonium Super Phosphate.

Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (smith) [29] is an important soil-borne disease that affects a wide range of staple and cash crops [12]. Application of organic amendments is an effective measurement for controlling soil born disease [1-2, 9, 16]. The amendment of soil with organic matter increases the soil biomass and can enhance the activity of specific antagonists within the community of soil microorganisms [11, 15]. The early attempt to reduce damage from bacterial wilt by soil amendment with fertilizers and organic matter were not very successful with the exception of the treatment with urea [19]. After adding several organic matters to soil [6], only shrimp shell powder and urea reduced rapidly the population of *R.solanacearum* and thus prevented the development of bacterial wilt of tomato under greenhouse conditions. A soil amendment, called S-H mixture (mainly consisted of agriculture and industrial wastes) was effective in reducing the incidence of many diseases in field or greenhouse trials and [5, 7, 18, 22, 27-28, 30]. The present work aimed to study the effect of organic matters and mineral fertilizers on the population of virulent and a-virulent forms of *R.solanacearum* on suppressing the potato bacterial wilt disease under artificial inoculation conditions.

Material and Methods

A. Inoculum preparation and soil infestation

Virulent isolates of *R.solanacearum* was isolated and identified from infected potato tubers. The bacterium was grown on Sucrose Peptone Agar (SPA) medium at 28°C for 48hr. Bacterial growth was suspended in Sterilized Distilled Water (SDW) and adjusted according to its optical density to 600 nm with 0.03 to introduce 3.2 x 10^7Colony Forming Units (CFU/ml) according to [23]. Soil infestation was carried out by adding 200 ml of bacterial suspension per kg soil. Infestation procedure was made 48hr before plants.

B. Tubers and sawing

Potato tubers (Diamont, cv), were stored at 4°C for the dormancy break stage. Then, it placed in trays at room temperature "in the dark" to stimulate germination. Within 2-3 weeks; tuber pieces contained one or more sprouts. Germinated tubers (each piece containing 2 sprouts) were planted in plastic trays (45 x 30 x 30 cm) containing sterilized sandy-clay soil (1:1, v: v) which previously infested with the pathogen.

Two tuber pieces were planted per tray. Four trays were used as replicates for each treatment. These trays were irrigated by equal intervals.

C. Treatments

Dry ground leaves of cabbage, camphor and garlic were applied alone at rates 0, 1, 2 and 3 g/kg soil as organic matters. Ammonium nitrate (33.3% Nitrogen), Ammonium Super-Phosphate (46% P_2O_2 + 16% Nitrogen) and Potassium Sulfate (52% KO_2 +17- 18% Sulfate) were applied alone at rate 0, 1, 2 and 3 g/kg soil; as mineral fertilizers. Both organic matters and mineral fertilizers were added; twenty days; before planting. The organic and mineral amendments were very well mixed with the soil.

D. Determination of *Ralstonia solanacearum* population in potato rhizosphere

Ten grams of potato rhizosphere soil per each treatment was tested at 0, 20, 40, 60 and 80 days from planting. Each sample was placed in flask (250 ml) containing 90ml of SDW and shaked for 30 min; using horizontal shaker. Serial dilutions were prepared and one ml of the 10^6 dilutions placed on Tetrazolium Chloride agar medium (TTZC) as selective medium to detect *R.solanacearum* [20].Virulent (fluidal, red colonies with broad white margins) and avirulent (afluidal, red colonies without broad white margins) colonies were estimated after 3 days of incubation at 28°C to a proportion of one gram rhizosphere soil. Three plates were used as replicates for each treatment.

E. Disease assessment

Disease severity was assessed 90 days after planting time. Potato bacterial wilt disease severity was evaluated as percentage of wilted plants; which are showing typical wilt symptoms; in relation to the total plants. Moreover, disease severity as disease index (%) was calculated from disease rating; for individual plants [21]. However, scale of determination was based on the visual observation of foliage wilt (zero= no symptoms, 1= symptoms up to 25%, 2= 26 -50%, 3= 51-75%, 4=76 -100% and 5= dead plants). Disease index (*D.I*) was calculated by the following formula: $D.I = \frac{\sum R.T \times 100}{S \times N}$, where T= total number of plants in each category, R=Disease severity scale R (R=0, 1, 2, 3, 4, and 5), and N= total number of plants tested. Also percentage of disease reduction (P.D.R) was calculated from percentage of wilted plants as following formula: $P.D.R = \frac{WPcr - WPtr \times 100}{WPcr}$, Where, WP_{cr}= Percentage of wilted plants in control treatment. Data were statically analyzed using the "F" test and the value of L.S.D ($P<0.05$) was calculated according to [8].

Result

Effect of organic matters and mineral fertilizers, under artificial inoculation conditions on

A. Population of *R.solanacearum* in potato rhizosphere soil

The obtained data were illustrated in figure (1). Addition of organic matters and mineral fertilizers as soil amendment to the soil led to population reduction of virulent forms of *R.solanacearum*, while, avirulent forms were increased as compared to the control treatment. Increasing the doses of soil amendment introduce high effectiveness on virulent or avirulent forms of *R.solanacearum* population. Furthermore, period of 20 to 40 days and/or 40 to 60 days promote the bacterium appearance of avirulent form when organic matters or the mineral fertilizers were applied, respectively. In addition, application of dry ground leaves of garlic as organic matters was more effectual than the dry ground leaves of cabbage and camphor. Garlic dry ground leaves decreased the virulent forms of *R.solanacearum* among population density of the bacterium with a virulent form. Meanwhile, potassium sulfate as mineral fertilizer was more effective than ammonium nitrate and ammonium super-phosphate.

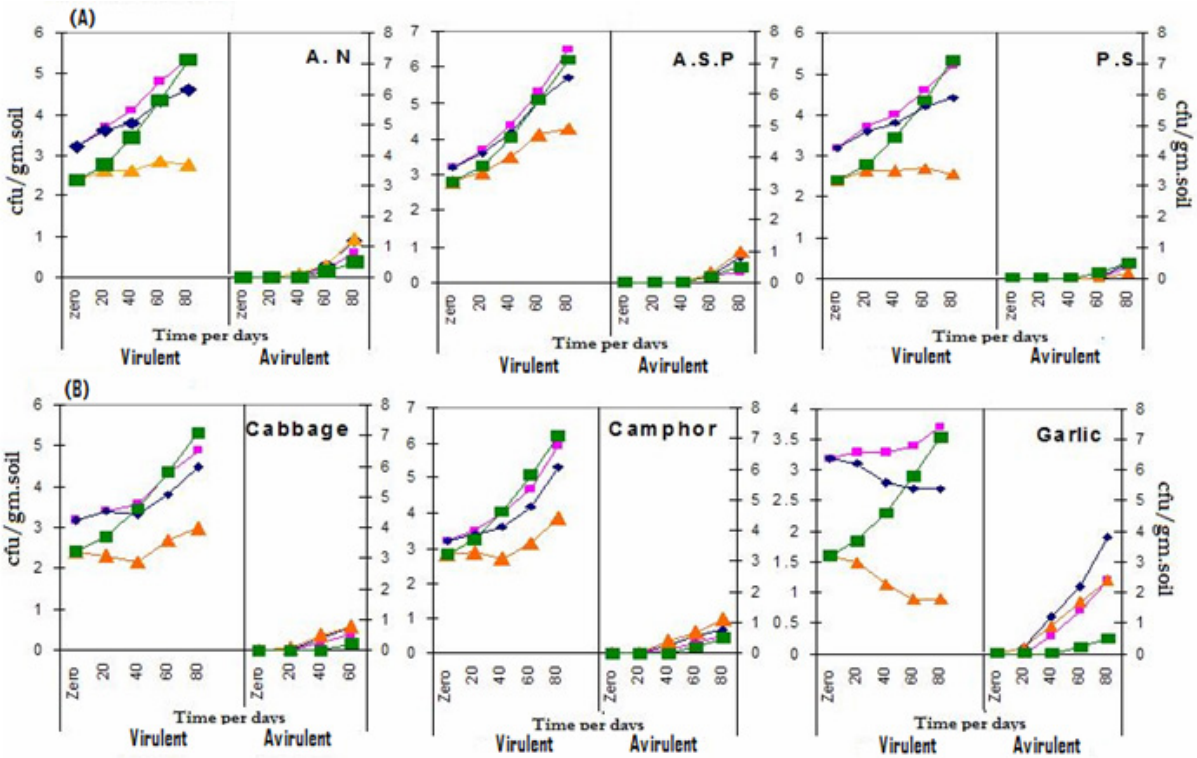

Figure (1) Influence of different **(A)** chemical fertilizers; Ammonium Nitrate (A.N), Ammonium Super-Phosphate (A.S.P), Potassium Sulfate (P.S) and **(B)** organic matters (Cabbage, Camphor, Garlic), at different application rates (zero, 1, 2, 3 g/plant) on population of virulent and avirulent forms (cfu/gm.soil) of *Ralstonia solanacearum* in potato rhizosphere soil, under artificial conditions.

B. Severity of potato bacterial wilt

Data in figure (2) indicated that disease severity was decreased when organic matters or mineral fertilizers applied as soil amendment in comparison with the control. Practically, application of the garlic dry ground leaves; significantly reduced the disease severity, where percentage of disease reduction was 62.1-77.7%. Meanwhile, application of cabbage or camphor dry ground leaves was moderately effective. Furthermore, percentage of the disease reduction was 49.5-64.5% and 38.6- 51.6%, respectively. As a result, disease severity was reduced through increasing the soil amendment used. On the other hand, application of potassium sulfate as mineral fertilizer was more useful than ammonium nitrate and ammonium super-phosphate, where percentage of disease reduction was 45.7-64.2%, 31.1-47.0% and 29.4-43.6%, respectively.

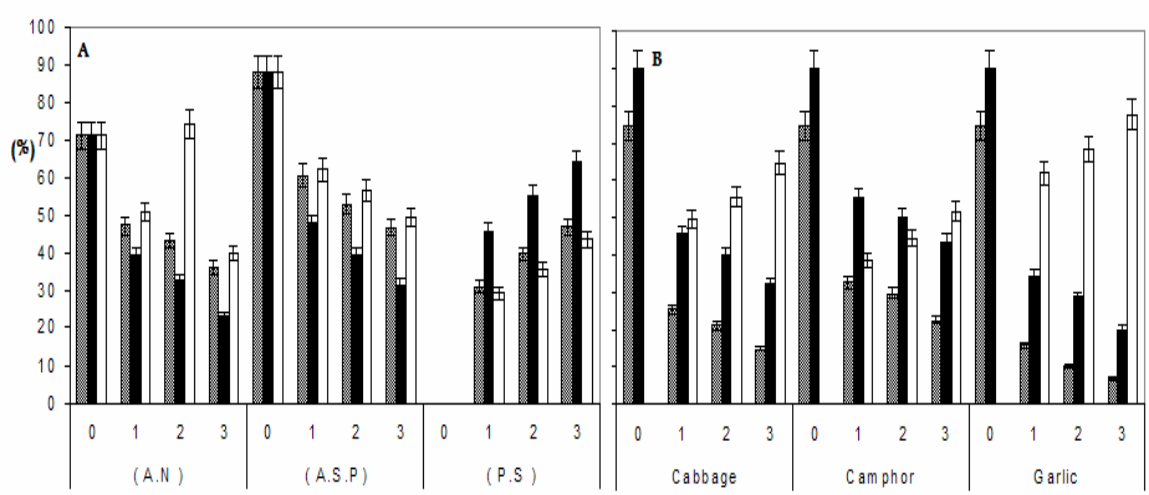

Figure (2) Influence of different **(A)** Chemical fertilizers; Ammonium Nitrate (A.N), Ammonium Super-Phosphate (A.S.P), Potassium Sulfate (P.S) and **(B)** organic matters (Cabbage, Camphor, Garlic), at different application rates (zero,1, 2, 3 g/plant) on severity of bacterial wilt of potato as Disease Index (D.I), percentage of Wilted Plants (W.P), and percentage of Disease Reduction (D.R) under artificial conditions.

Discussion

Soil treatment with organic matters and/or mineral fertilizers have been influenced the presence of *R.solanacearum* population density forms; virulent among a virulent form in potato plants soil rhizosphere; as compared to the control treatment. Increasing the rate of the tested compounds enhanced their effectiveness on the population of the bacterium and the disease severity. Application of garlic dry ground leaves as organic matter and potassium sulfate as mineral fertilizer; shows great impact against the virulent forms of *R.solanacearum* population and disease severity. Consequently, the result are in agreement with [17], who concluded that organic amendment influenced the soil born disease severity by (1) increasing the biological buffering capacity of the soil, (2) reducing pathogen numbers during the anaerobic decomposition of organic matter and (3) nitrification process influences the dominant form of the nitrogen (N_2) in soil rhizoshpere . Moreover, (26) recorded that organic soil amendment leads to physical possessions on soil properties including (a) reducing the bulk density of the soil, (b) increasing water holding capacity, (c) increasing water infiltration and drainage in fine- textures and (d) improving of the soil aggregation. Application of soil amendment and mulches has beneficial influence on crop health. Therefore, percolation of water is improved to greater depth; lessening crust formation or releasing mineral nutrients. On the other hand, there is a great impact on plant diseases through enhancement of antagonistic activities among the wilt disease occurrence of many crops [13-14]. Disease severity incidence was reduced with application of organic amendments due to the release of toxic NH_3 or increasing the resident levels of antagonist [25]. Pathogen population was decreased after adding urea as soil amendment due to accumulation of ammonium or nitrate which was formed from the decomposition and toxicity, and furthermore, it increases the soil pH value. Bacterial wilt of tomato was significantly decreased with application of urea or calcium oxide due to their effect on population dynamic of the pathogen and on soil pH value [10]; [23-24]. Cruciferous plants are known to contain glucosinolates, i.e. sulfer-containing secondary metabolites, which are hydrolyzed to produce IsoThioCyanates (ITCs). The I.T.C.S exhibits inhibitory effects on a wide variety of microbes [3]. Inhibitory effects on plant pathogens following the incorporation of cruciferous plant residues into soil have been attributed to the "biofumigation" by toxic volatile compounds released by degradation of glucosinolates [4].

Acknowledgments The author wish to thank professor dr. Abd El-Gafar, N.Y. Plant Disease department, Faculty of Agriculture, Ain Shams University; for supervising and always supporting this research study.

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Viability of culturable soil microorganisms during freeze storage

A. Muñoz González[*,1], A. López Piñeiro[2], and M. Ramírez Fernández[1]

[1] University of Extremadura, Area of Microbiology. Avda Elvas s/n, 06071 Badajoz, Spain.
[2] University of Extremadura, Area of Edafology.

[*]Corresponding author: e-mail: anamg@unex.es , Phone: +34 924289426

Soil microbial populations are preferably studied by analysing fresh soil samples, but this may not always be feasible in field conditions. We therefore studied the effect of freeze storage (at -20°C, up to 44 months) on soil culturable microorganism viability. Most culturable soil bacteria were not affected by the prolonged freeze storage and freeze-thaw stress, but the viability of the fungus and sporulating bacillus populations significantly decreased after 8-12 months frozen. These changes did not, however, significantly affect the total culturable microorganism count, biodiversity index, or differences in biodiversity between soils. Therefore, freeze storage for up to 8 months may be allowed for analyses of culturable microbial population biodiversity, and longer times may be reasonably acceptable.

Keywords soil microorganism viability; freeze storage; freeze-thaw stress.

1. Introduction

When the immediate assay of soil samples is unfeasible, recommendations for soil handling often include refrigeration or freezing in the interim between collection and assay, even if the storage period is likely to be short [1]. The ISO standard [2] explicitly warns against freezing as a soil storage method for the laboratory assessment of aerobic microbial processes. On the contrary, the OECD guideline [3] suggests that soils can be stored frozen (-20º ± 2ºC) for 1 year. In both of these sources, the recommended temperature for soil sample storage is +4ºC for a maximum of 3 months [2,3], but some studies have shown that freezing is preferable to cold storage [4].

The effect of freeze-thaw stress on soil microbial populations has been studied by focusing on particular activities such as methidathion degradation [5], nitrification and denitrification [6-8], and hydrocarbon biodegradation [9]. Also, the microbial biomass of soil has been measured by the substrate-induced respiration (SIR) method [10] after frozen and cold storage. All these studies have found that freeze-thaw stress does affect soil microbial populations, reducing some biological activities [11], increasing others [8], or reducing certain specific microbial communities [12, 5].

In many soil microbial studies, some analyses are impossible to perform immediately after sampling, so that some form of storage is essential. We therefore here evaluated the effects of freeze storage and thawing on the microbial population biodiversity.

2. Material and Methods

2.1 Soil sampling and analysis

Samples were collected from the surface layer (0-10 cm depth) of three agricultural soils of maize crop located in south-western Spain: soil 1 (under conventional management), soil 2 (direct seeding), and soil 3 (direct seeding with a cover crop). The three soils had low K and very low pH. They mainly differed in total organic carbon content (10.3, 13.5, and 28.3 g kg^{-1}), volumetric soil water content (0.21, 0.27, and 0.31 cm^3 cm^{-3}), total N (1.21, 1.26, and 2.27 g kg^{-1}), and available P (56.4, 40.7, and 125.9 mg kg^{-1}) for soils 1, 2, and 3, respectively. Samples were taken every 2 months over three consecutive years. They were passed through a 2 mm sieve, and a time zero microbial population analysis was performed (see below). Then 50 grams aliquots were quickly (less than 5 min) freeze stored at -20ºC. All the aliquots were thawed (at room temperature) 44 months after the first sample storage. Using this approach, one is able to analyse at one time the effect of freeze storing and thawing on the viability of the soil microorganisms present in a given soil at different times over a long period. Properly diluted soil aliquots were inoculated on two rich culture media - TSA (Tripticase Soy Agar) and YEPD (Yeast Extract Peptone Dextrose) - for the microbial population analysis. The plates were incubated at 25 ºC up to 10 days to detect slow-growth microorganisms. A total of 26 bacterial morphological types were detected by analyzing colony morphology, cell shape (microscopy), sporulation capability, and Gram staining (Table 1).

Table 1 Bacterial morphological types detected in rich culture media.

Type	Colony morphology					Cell shape	Sporulation capability	Gram staining
	Form	Margin	Texture	Opacity	Color			
T1	Irregular	Undulate	Shiny	Opaque	Orange	Cocci	No	-
T2	Circular	Entire	Shiny	Opaque	Yellow	Rods	No	+
T3	Irregular	Entire	Mucoid	Translucent	Beige	Rods	No	-
T4	Circular	Curled	Wrinkled	Opaque	Violet	Rods	No	-
T5	Circular	Entire	Shiny	Opaque	White	Cocci -rods	No	+
T6	Irregular	Entire	Smooth	Opaque	Beige	Rods	No	-
T7	Irregular	Lobate	Mucoid	Translucent	Beige	Rods	No	+
T8	Circular	Entire	Shiny	Opaque	Beige	Rods	No	+
T9	Circular	Entire	Rough	Opaque	Beige	Cocci	No	+
T10	Circular	Entire	Rough	Opaque	White	Rods	No	-
T11	Irregular	Undulate	Dull	Opaque	Red	Cocci -rods	No	+
T12	Circular	Entire	Mucoid	Translucent	Yellow	Cocci	No	-
T13	Irregular	Entire	Rough	Opaque	Orange	Cocci -rods	No	-
T14	Circular	Entire	Smooth	Opaque	White	Cocci	No	-
T15	Circular	Entire	Dull	Opaque	Yellow	Rods	No	-
T16	Irregular	Lobate	Shiny	Transparent	-	Rods	No	-
T17	Circular	Entire	Shiny	Transparent	-	Rods	No	+
T18	Irregular	Entire	Mucoid	Opaque	Beige	Rods	No	+
T19	Circular	Entire	Shiny	Opaque	Pink	Rods	No	-
T20	Circular	Entire	Mucoid	Opaque	Brown	Cocci -rods	No	-
T21	Rhizoid	Curled	Wrinkled	Opaque	Beige	Large rods	Yes	+
T22	Circular	Entire	Mucoid	Traslucent	Beige	Cocci -rods	No	-
T23	Irregular	Lobate	Mucoid	Traslucent	Beige	Rods	No	-
T24	Circular	Entire	Shiny	Opaque	Brown	Rods	No	-
T25	Circular	Entire	Mucoid	Opaque	Yellow	Rods	No	-
T26	Irregular	Entire	Granular	Opaque	White	Rods	No	-

2.2 Statistical analysis

Simpson's reciprocal index of diversity [13] was used as a measure of the soil microbial diversity. The data were subjected to analysis of variance (ANOVA) and comparison of means by the Duncan test (at $P < 0.05$).

3. Results and Discussion

Most bacterial populations were not affected by the freezing time or the freeze-thaw stress: the number of colonies remained the same after 44 months freeze storage for 25 out of the 26 morphological types analyzed (Fig. 1C). The exception was the sporulating Bacillus mycoides, extensively present in the three soils studied, which showed a decreasing trend with the freezing time (Fig. 1B) down to 25% of the original colony number. Similarly, the fungus populations decreased progressively after 12 months of freeze storage, becoming undetectable after 44 months (Fig. 1A).

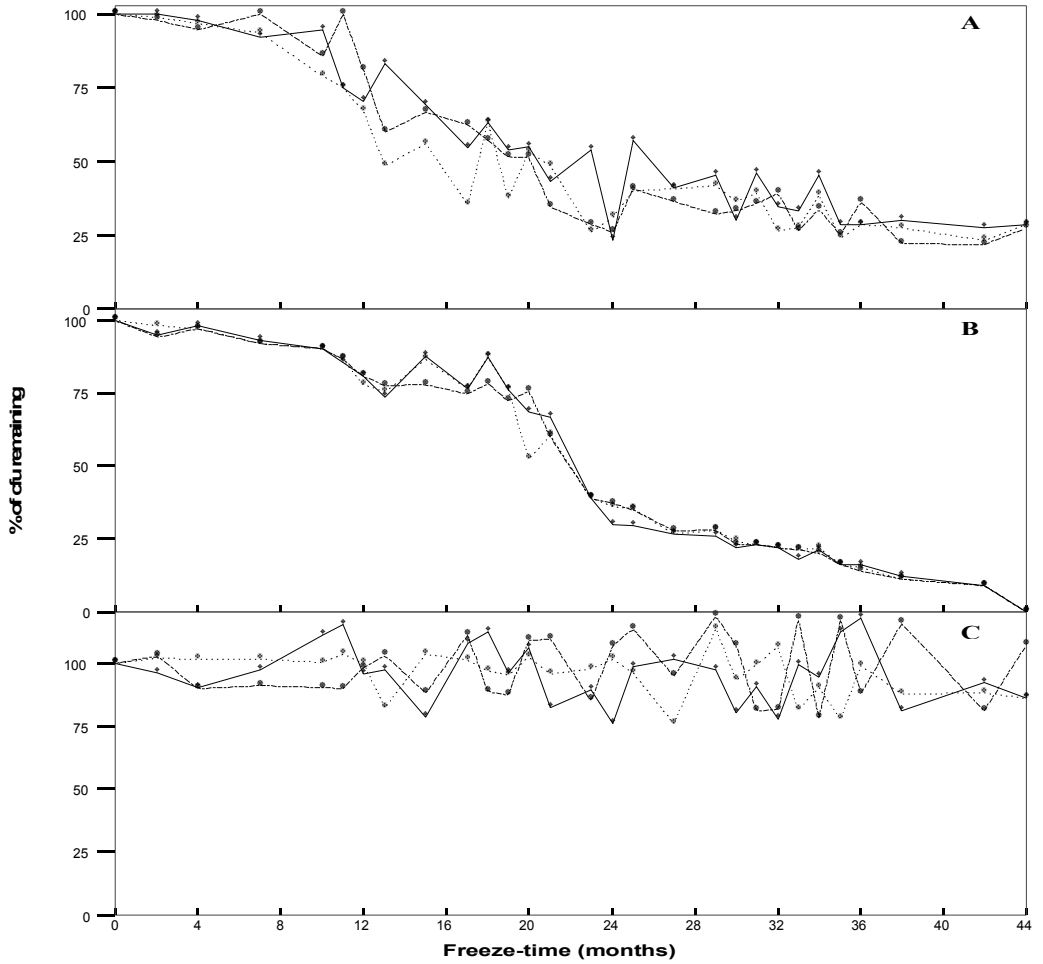

Fig 1. Effect of freezing time and thawing of soil samples on (A) fungus populations, (B) *Bacillus mycoides*, and (C) the rest of the bacterial populations. Colony counts (cfu) from soil 1 (●), soil 2 (▲), and soil 3 (■).

The loss of viability of *Bacillus mycoides* and fungus populations may be because they have no time to sporulate before freezing, with the vegetative cells being more sensitive to freeze storage than their spores or the rest of the bacteria. The live-microorganism decreasing trend was similar in the three soils for both the *Bacillus mycoides* and fungus populations ($P > 0.05$), independently they have differed total organic carbon content.

Despite the observed decreasing trend of the cell viability of *Bacillus mycoides* and the fungi, they both represented only minority amounts in the total microbial populations of these three soils (<1% and <2% respectively). On the contrary, the rest of the bacterial populations, which represented the great majority, did not change throughout the freeze storage time. While other workers have found an irreversible decrease in the DNA content and direct cell counts after freezing and thawing (Morley et al., 1983; Pesaro et al., 2003), which they explain as being accountable for by changes in the bacterial populations, they were unable to detect such changes using the domain-specific PCR-RFLP fingerprints, excepting for a decreasing trend in some Archaea communities [5].

Consequently, the observed slight decreases in the Simpson's diversity index of the total microbial populations after 44 months of freeze storage (Fig. 2) were never statistically significant ($P > 0.05$). It is interesting that the greater the microbial population diversity, the smaller was the apparent decrease (Fig. 2).

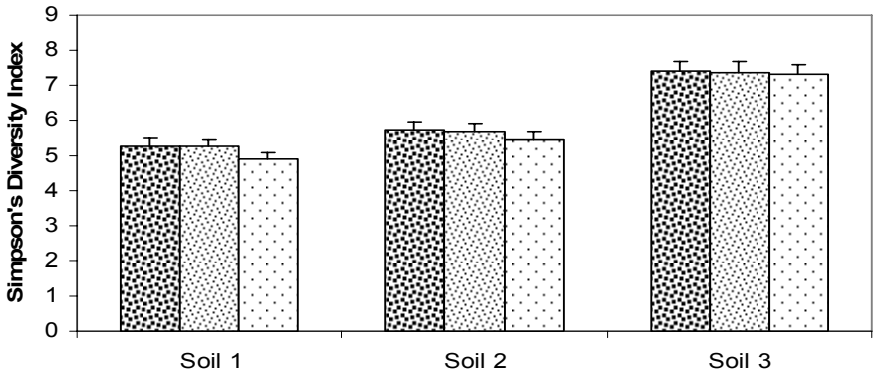

Fig 2. Simpson's diversity index of total microbial populations of the soils, before freeze storage (▩), after 8 months of freeze storage (▩), and after 44 months of freeze storage (▩).

4. Conclusions

In conclusion, -20ºC freezing is suitable for the storage of the most abundant non-sporulating bacteria of the three soils studied. However, it is not appropriate for preserving the fungus and *Bacillus mycoides* populations for longer than 8 months. Therefore, freeze storage for up to 8 months may be allowed in analyses of microbial population biodiversity, and longer times could be reasonably acceptable because the diversity indices of the total microorganism population were not significantly affected.

Acknowledgements The support by the Spanish Ministry of Science and Technology (project AGL 2000-0463-P4-05) and the Extremadura Regional Government (project 2P04B003 and Ana Muñoz's grant) is gratefully acknowledged.

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Analytical and Imaging Techniques.

Microscopy

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Characterization of Cell Walls from Mucoralean Fungi by Biochemical Composition, Transmission Electron Microscopy and X-Ray Microanalysis

G. M. Campos-Takaki[1] and Sônia M. C. Dietrich[2]

[1] UNICAP, Centro de Ciências e Tecnologia, Núcleo de Pesquisas em Ciências Ambientais, Recife, Pernambuco, Brasil E.mail: gmctakaki@pesq.cnpq.br
[2] Instituto de Botânica de São Paulo. Água Funda 04301-902 - São Paulo, SP, Brasil. pgibt@ibotsp.gov.br

The cell walls isolated from the mycelia of Mucoralean fungi are largely constituted of microfibrillar fraction bound together by proteins, lipids, polyphosphate, and a variety of polysaccharides. The fibrillar fraction corresponds to chitin. Chitosan, is originated from chitin, and is a nontoxic copolymer. The objective of this paper was to investigate the biochemical composition of the cell walls of *Absidia cerulea, Mortierella alpina, Mucor mucedo, Rhizopus arrhizus*, and *Syncephalastrum racemosum*. The results showed the cell walls are largely constituted by carbohydrates, and the chitin microfribilles organization was characterized by transmission electron microscopy. The X-ray microanalysis showed the presence of Ca^{+2} linked to the microfibrillar fraction, and that association was demonstrated for the first time in cell-walls of Mucoralean fugi.

Keywords Mucoralean fungi, Cell walls, Characterization, X-ray microanalysis, Transmission Electron Microscopy

1. Introduction

Typically, the cell walls contain microfibrillar materials bound together by proteins, lipids and a variety of polysaccharides. While the fibrillar material, chitin, of the wall is largely inert, the composition of the inserted materials changes with time. Most fungi have a fibrillar structure built on chitin, and chitosan, only Zygomycetes[1]. Chitin is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose. Chitin has great economic value as due to their versatile biological activities and chemical applications, mainly in the medical and pharmaceutical industries, and in dealing with environmental contamination [2]. The polysaccharide is found in a wide range of many sources, such as crustaceous, insect annelids, mollusks, coelenterates and is a common constituent of fungal cell walls, in special Mucoralean fungi [3]. Chitin, is insoluble linear β1,4- linked homopolymer of N-acetyl-D-glucosamine (GlcNAc), and it is the second most abundant polysaccharide, after cellulose. Chitosan is a cationic amino polysaccharide, essentially constituted of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues, derived from de-*N*-acetylation of chitin [4]. It has become of great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields, and recent progress in chitin chemistry is quite noteworthy. The main advantage of chitosan over chitin is that it is soluble in dilute acid solutions through protonation of amine groups.

The development of applications for chitosan has expanded rapidly in recent years. Chitosan, being polycationic, nontoxic, biodegradable as well as antimicrobial, has many applications especially in the agriculture, food and pharmaceutical industries. Chitin and chitosan have a very diverse range of applications such as in the food, textile and cosmetics industries in addition to environmental and biomedical applications [5]. Chitin, and especially chitosan have been used as bone and tooth filling material, drug conjugates, microparticulate controlled drug delivery systems, enzyme immobilization, wastewater treatment, as food additive and anticholesterolemic, for wound healing, and in pharmaceuticals in several drug delivery systems. The ability of chitosan to chelate metal ions both in solution and in the solid state have been studied, suggesting the possibility of using this polymer in wastewater treatment for environmental purposes. However, no reports in the literature could be found on any biomedical application of the ion-chelation capacity of chitin or chitosan [6,7,8,9].

The aim of this study was to investigate the cell walls content of the polysaccharides chitin and chitosan, proteins, lipids, inorganic polyphosphate, microfibrillar organization, and X-ray microanalysis in *Absidia cerulea, Mortierella alpina, Mucor mucedo, Rhizopus arrhizus*, and *Syncephalastrum racemosum*.

2. Material and methods

Microorganisms: In this work were used Mucoralean fungi as: Absidia cerulea, Mortierella alpina, Mucor mucedo, Rhizopus arrhizus, and Syncephalastrum racemosum. The strains belong to the culture collection of Catholic University of Pernambuco, maintained on potato dextrose agar and Sabouraud dextrose agar at 5ºC.

Microbiological cultivation: Spores 107/mL of the microorganisms were used for fungal submerse cultivation in Erlenmeyer's flasks of 1000mL containing 300mL of Hesseltine and Anderson medium, modified by Campos-Takaki at al. [9] incubated at 25ºC in an orbital shaker at 150 rpm for 96 hours. The mycelia were collected by filtration, washed with deionizer water, and were lyophilized. The dried biomass was submitted to cell walls were prepared and purified.

Biochemical composition of the cell walls

Chitin and chitosan: The mycelium of the strains *Absidia cerulea, Mortierella alpina, Mucor mucedo, Rhizopus arrhizus*, and *Syncephalastrum racemosum* were submitted to fractionation and differential extraction using 2N NaOH at 23°C, during 12 hours, followed centrifugation at 2000 *g*. The residual fraction was washed 3 times with distilled water, and treated with 1N H_2 SO_4 at 96°C during 16 hours, followed centrifugation at 2000 *g,* the supernatant was of neutralized and precipitated with three volumes of ethanol, and maintained at 5°C overnight. The residue was extracted using 2N NaOH at 23°C, during 39 min, followed centrifugation at 2000 *g*. The residual fraction was washed three times with distilled water, followed dialysis against distilled water during 24 hours, corresponding to alkali-acid insoluble fraction chitin [10].
Protein: The total protein was extract from mycelium submitted to distilled water followed maceration and centrifugation at 3000 *g*. The supernatant was used to total protein determination by Lowry method [11], using albumin serum bovine as standard.
Inorganic polyphosphate: The content of inorganic polyphosphate in the cell walls was determinate by Rouser et al. [12]. The hydrolysis was done using perchloric acid at 70%, at temperature of 180 °C, during 20 min. After two hours were added distilled water, and the color reagent, followed of 5 min at 37 °C. The spectrophotometer determination was done at 797 nm using monobasic potassium phosphate solution as standard.
Lipids: The lipids content were determined according to Manocha et al. [13], using chloroform and methanol (2:1, 1:1, 1:2 v/v). The analysis was carried out using gas liquid chromatography, column SP 2340, at temperature of 150 °C, vaporization 260 °C, nitrogen flux 33.4mL/min. The identification was done using lipids standard retention time.
Transmission Electron Microscopy: The microfibrillar fraction was obtained by Hawes method [14] using H_2O_2 and acetic acid (1:1 v/v) submitted to 5 hours at 100 °C, followed wash treatment and the suspension was deposited grid, and dried at 37 °C. The grids were covered using formvar solution, and dried at 37 °C. The grids were observed by transmission electron microscopy 6B, Zeiss.
X-Ray microanalysis: The samples after the preparation to microfibrillar fraction were observed by transmission electron microscopy, operator at 60 KV, using a detector of dispersive energy (X-ray) REVEX, associated to analyzer calcium and phosphorus. The window was fixed in 1.88 to phosphorus and 2.14 to calcium. The aperture was fixed in 14.0-16.0 KeV, using the formulation described according Appleton [15]. The author describes the use of that formulation it is possible the comparison of the peaks of calcium and phosphorus in different samples.

3. Results and discussion

3.1 Biochemical composition of cell walls of Mucoralean fungi

Zygomycota, like all true fungi, produce cell walls containing chitin. They grow primarily as mycelia, or filaments of long cells called hyphae, and it is the most ecologically diverse group of fungi, functioning as saprophytes on substrates. However, some species have a negative economic impact on human affairs by causing storage rots of fruits (particularly strawberries by *Rhizopus stolonifer*, as agents of plant disease (e.g., *Choanephora cucurbitarum* flower rot of curcurbits), while other species can cause life-threatening opportunistic infections of diabetic, immuno-suppressed, and immuno-compromised patients [16, 17].

The cell walls obtained from the mycelia of Mucoralean fungi was constituted by proteins (4.5 to 10.4%), polyphosphate (8.6 to 21.0%), and lipids (6.4 to 15.7%). Differential extraction of cell walls distinguished the polysaccharides chitin and chitosan, proved to be the most abundant content. The fractionation of the polysaccharides provided evidence the existence of a glycan component linked to chitin. This fraction consisted of a galactose homopolymer. However, the higher components of the cell walls of Mucoralean fungi are carbohydrates, in special, chitin/chitosan (Table 1).

Table 1 – Biochemical composition of the cell walls of Mucoralean fungi

Strains	Protein %	Carbohydrates Not aminated %	Chitin/ Chitosan %	Poly P %	Lipids %	Total %
(A) (D) Absidia cerulea	**4.5**	**18.0**	**37.0**	**20.4**	**15.7**	**94.6**
(B) Mucor mucedo	**10.4**	**22.9**	**42.0**	**13.1**	**9;1**	**97.5**
(C) Rhizopus arrhizus	**10.2**	**14.7**	**43.0**	**18.6**	**8.6**	**95.1**
(E) Mortierella alpina	**9.0**	**18.0**	**40.8**	**21.0**	**10.9**	**98.9**
(F) Syncephalastrum racemosum	**10.1**	**22.4**	**44.0**	**8.6**	**6.4**	**96.8**

Those results are corroborated by Bartnicki-Garcia and Lippman [18]. The inorganic polyphosphate (poly P) is a linear polymer of phosphoanhydride-linked phosphate residues that occurs in the cell walls of Mucoralean fungi. It was found particularly abundant in the fungal cell wall of A. cerulean and M. alpine, following R. arrhizus,, M. mucedo, and S. racemosum (Table 1). On the other hand, the cytochemistry and electron microscopy results indicated the presence of polyphosphate on the outer most surface of the cell wall contain poly P in the outer layer, but very little is known about the nature and functions of poly P in this compartment. Mucorales showed granules of Poly P in the outer layer of cell walls. The higher presence of Poly P it is a characteristic of that fungi than the other fungi described by the literature [19, 20].

The results indicated for the first time by X-ray microanalysis the presence of Ca+2 biding to microfibrillar chitin fraction. The last layer of the cell wall showed the presence of Mg+2, P (phosphorus) S (sulfur), Cl (chloro), and K (potassium), too. All elements were detected in the last layer of the cell wall. The presence of S (sulfur) is suggested to a protein containing amino acids as tyrosine linked to chitin fraction[21] (Table 2).

Table 2 – Elements detected in the cell walls from chitin fraction of Mucoralean fungi

Strains	Mg	P	S	Cl	K	Ca
(A) (D) Absidia cerulea	-	+	+	-	+	+
(B) Mucor mucedo	-	+	-	+	-	+
(C) Rhizopus arrhizus	-	+	+	-	-	-
(E) Mortierella alpina	-	+	+	+	+	+
(F) Syncephalastrum racemosum	+	+	+	+	+	+

The transmission electron microscopy of the microfibrillar fraction of the cell walls of Mucoralean fungi was observed in the Figure 1. The microfibrilles of chitin were randomic distributed in the last layer (Fig. 1-E). The apical region of the hyphae of *Absidia cerulea* showed a thin layer of microfibrillar fraction of chitin, emerged in protein, and it was a characteristic of extension region of the hyphae.

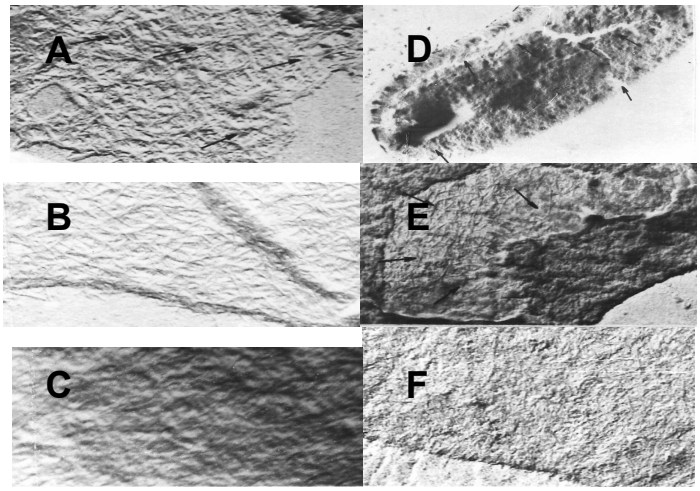

Figure 1. Transmission Electron Microscopy of the microfibrillar fraction (chitin) isolated from Mucoralean fungi. A. *Absidia cerulea*; B. *Mucor mucedo*; C. *Mortierella alpine*; D. Apical region of *Absidia cerulea*; E. *Rhizopus arrhizus*, and F. *Syncephalastrum racemosum*. 30000x

4. Conclusions

In conclusions the cell walls obtained from the mycelia of Mucoralean fungi selected were constituted by amounts of proteins, polyphosphate, lipids, and polysaccharides. The differential extraction of cell walls distinguished the polysaccharides chitin and chitosan, proved to be the most abundant content. The fractionation of the polysaccharides provided evidence for the existence of a glycan component linked to chitin. That fraction consisted of a galactose homo polymer. The cytochemistry and electron microscopy indicated the presence of polyphosphate on the outer most surface of the cell wall. And, the x-ray microanalysis showed the presence of Ca^{+2} linked to microfibrillar fraction (chitin). In addition, the study realized with Mucoralean fungi contributed with the acknowledge of fungal biology, and biochemistry of the versatile contents obtained from cell walls.

Acknowledgements- The authors are grateful to the Brazilian agencies CNPq, CAPES,, FACEPE for financial support.

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Comparative analysis of different microbial techniques of quantification applied to anaerobic digestion

B. Montero*, J.L. García-Morales, D. Sales, and **R. Solera**

Department of Chemical Engineering, Food and Environmental Technologies, Faculty of Marine and Environmental Sciences, University of Cadiz, Campus Rio San Pedro s/n, 11510 Puerto Real, Spain

*Corresponding author: e-mail: blanca.montero@uca.es, Phone: +34 956016157

In the processes of high microbiological complexity and operating extreme conditions, as thermophilic-dry anaerobic digestion, the application of specific microbiological techniques is the principal tool to accomplish the start-up and control of the equipments. The estimate of microorganisms number in mixed cultivations is very complex, since the diversity of these populations is extreme. Therefore, it is convenient to use several procedures of quantification. DAPI technique is representative of main microbial groups contained in the digester in each organic loading rate studied. While than autofluorescence microscopy technique was not representative of the total methanogenic population or specific. Therefore, the utilisation of a specific technique to determine the methanogens was necessary. FISH technique has allowed to quantify total methanogenic population or specific (H_2-utilising methanogens and acetate-utilising methanogens) by ARC915, MB1174 and MX825 probes, respectively.

Keywords anaerobic digestion, autofluorescence, DAPI, fluorescent in situ hybridization, municipal solid waste

1. Introduction

Anaerobic digestion is a process of microbiological character that the organic matter, in the absence of oxygen, turns into stable and inert final products and biogas (methane and carbon dioxide), susceptible of use and economic evaluation. Normally, the control of anaerobic digestion is carried out by the application of physical-chemical techniques. These do not provide information on the composition and the physiological state of the biomass contained within the system [1]. Consequently, and in order to acquire more detailed information in respect of this biomass, other parameters have been used in the quantification of the microorganisms involved in the anaerobic process.Epifluorescence microscopy with fluorometric stains, in particular 4´,6-diamidine-2-phenylindole (DAPI), are widely used for direct counting of total microorganisms [2-4]. A characteristic peculiarity of methanogens is their UV-induced blue-green autofluorescence which permits counting by autofluorescence microscopy [4, 5]. However, this method is subjective under following conditions: it only shows methanogens with a high content of F_{420} such as hydrogen-utilising methanogens; acetate-utilising methanogens belonging to the genus *Methanosaeta* can not be counted at all and genus *Methanosarcina* are found in clumps made up of many individual cells. Nevertheless, it has been one frequently used method to count autofluorescent methanogens in anaerobic reactors until recent years [6, 7].

There are, however, several drawbacks to direct observational methods, including the inability to distinguish living from dead microorganisms and the inability to perform further studies on the observed microorganisms. Because of this, an important avenue of research has been the development and utilization of molecular techniques. Molecular techniques have successfully been applied for the direct detection and identification *in situ* of individual microbial cells and have therefore been used to monitor the spatial distribution of microorganisms in environmental samples and treat system. However, it must be kept in mind that the physiological state (living or non-living) is operationally defined and based on the general properties of a particular stain, and dormant or extremely slow-growing cells cannot be detected [3]. Nevertheless, DAPI epifluorescence method is usually used to obtain a total count even when molecular methods are applied [8]. Whole cell fluorescence *in situ* hybridization (FISH) is a technique that uses fluorescently labelled phylogenetic oligonucleotide probes to detect specific whole cells/organisms in biological samples. De Long *et al*. [9] first demonstrated its use with bacteria in 1989. It can be a valuable tool for the study of microbial dynamics in natural environments [10, 11]. For instance, it is possible to carry out a hierarchical phylogenetic analysis on a particular environment to identify the dominant groups of microorganisms present, after which temporal and spatial changes in the diversity and abundance of specific microbial population can be monitored in relation to environmental effects [8, 10-14]. Two good examples can be found in Sekiguchi *et al*. [15], who used FISH to study the morphology of the flocs in an UASB reactor, and Santegoeds *et al*. [16], who studied the morphology of aggregates present in three UASB lab-scale reactors. Raskin *et al*. [17-19] have used to identify and quantify species and genus of methanogens present in anaerobic reactors.

The main objective of this work was to compare the results obtained by the different microbial techniques of quantification applied to a thermophilic-dry anaerobic reactor during starting-up and stabilization stages.

2. Material and Methods

2.1 Experimental system

Anaerobic digestion of Organic Fraction of Municipal Solid Waste (OFMSW) was conducted in a laboratory-scale continuously stirred tank reactor (CSTR), with no recycling of biomass working in semicontinuous regime (one dose per day) in the thermophilic range (55ºC).The reactor was loaded with 1.5 kg of milled dry synthetic OFMSW (90% TS). The moisture was adjusted using an inoculum from SEBAC (Sequential Batch Anaerobic Composting) that consisted of a 1:1 v/v mixture of thermophilic sludge and leachate [20-22].A synthetic feed based on the nutritional requirements of the main populations of microorganisms involved was prepared [23]. The reactor was operated from 40 to 25 days of retention time (RT). The organic loading rate added to the system was modified, but a constant organic loading rate was maintained in each RT. It was used four organic loading rates (OLR): 4.42, 5.07, 5.92 and 7.50 kg volatile solid m^{-3} day.

2.2 Quantification techniques

Microbial numbers were determined by direct count procedures (microscopic counting methods). The total count was performed by the DAPI epifluorescence microscopy method [2,4]. Autofluorescent methanogens were counted by the autofluorescence microscopy method [4,5]. Main specific groups of microorganisms were quantified by Fluorescent *in situ* Hybridization (FISH), employing different oligonucleotide probes. The following 16S rRNA-targeted oligonucleotide probes were used in this study: *Bacteria*-universal probe EUB338 [24, 25], *Archaea*-universal probe ARC915 [26], H_2-utilising methanogens probe MB1174 (specifically *Methanobacteriaceae*) [15, 17, 18] that are the main hydrogen scavengers during start-up of thermophilic-dry anaerobic reactors [27] and acetate-utilising methanogens probe MX825 (specifically *Methanosaeta*) [17, 18]. The genus *Methanosaeta use* only acetate like characteristic substrate [17, 18, 27]. The biomass was determined by measuring volatile total solids (VTS). The relation between microbial quantification techniques were studied by Pearson correlation. The results that present a Pearson´s correlation coefficient higher than 0.8 were checked for linear regression with Spss v11.5 program.

3. Results and discussion

3.1 Comparative analysis of different microbial techniques of quantification

The results obtained by epifluorescence microscopy with DAPI, F_{420} autofluorescence microscopy and Fluorescent *in situ* hybridization using EUB338, ARC915, MB1174 and MX825 probes are shown in table 1. All the results shown are the average values for the total days of the study.

Table 1 Quantification of microbial population by different count techniques.

OLR_0 (gVTS $L^{-1}d^{-1}$)	DAPI (Cell $gVTS^{-1}$) ($x10^{-10}$)	Autofluorescence microscopy (Cell $gVTS^{-1}$) ($x10^{-7}$)	EUB338 (Cell $gVTS^{-1}$) ($x10^{-9}$)	ARC915 (Cell $gVTS^{-1}$) ($x10^{-9}$)	MB1174 (Cell $gVTS^{-1}$) ($x10^{-9}$)	MX825 (Cell $gVTS^{-1}$) ($x10^{-9}$)
4.42	2.06 ± 0.72	1.01 ± 0.83	0.93 ± 0.34	0.14 ± 0.08	0.25 ± 0.22	0.04 ± 0.06
5.07	1.10 ± 0.24	0.83 ± 0.52	1.45 ± 0.36	0.79 ± 0.42	0.41 ± 0.27	0.82 ± 0.42
5.92	1.34 ± 0.21	0.66 ± 0.21	1.46 ± 0.19	0.96 ± 0.21	0.25 ± 0.05	1.19 ± 0.18
7.50	2.06 ± 0.35	0.96 ± 0.34	2.64 ± 0.66	1.71 ± 0.37	0.43 ± 0.12	1.97 ± 0.60

The sum of EUB338 and ARC915 results is less than those obtained by DAPI. This could be due to the results obtained by FISH technique depend on personal skills and loss-free preparation. Glöckner *et al*. [28] estimated that approximately 10% of the cells were lost during preparation in comparison to a solely DAPI staining in parallel preparations. The most specificity of the oligonucleotide probes regarding the DAPI fluorochrome will bear to that some groups of microorganisms not dye for lack of affinity to the probes or for the complexity of this union. Moreover, it has been discovered that the EUB338 probe present three subtypes I,

II and III [29] and in this study it was only used a subtype. In this sense, it cannot be guaranteed that all the bacteria have been counted with this probe. Nevertheless, the difference is higher than 10%.

Furthermore, the variation in the proportion of cells detected using FISH may simply be the result of methodological artefacts with little or no ecological significance [30]. On the other hand, it is also possible that the proportion of cells that can be detected with oligonucleotide probes may be linked to variations in the physiological condition of the cells [31]. Several papers have shown that, at least in bacterial cultures, there is a link between the growth rate and the physiological condition of cells, their rRNA contents, and the detection of these cells using oligonucleotide probes [9, 32, 33]. The hypothesis that detection using the FISH protocol is directly related to the metabolic state of the cells, and therefore FISH may yield useful information on their physiology (e.g. in sediment) [34], has seldom been assessed for natural communities, but may explain some of the observed variability in the proportion of cells detected with this method.

In the same way, the obtained results by ARC915 probes are less than the sum of MB1174 and MX825. Since, the probes used to determine these methanogenic populations are more specific than ARC 915. Therefore, the results obtained present major affinity. However, the percentage of methanogenic obtained by sum is higher than autofluorescence microscopy. This could be due to autofluorescence microscopy can only be quantified the autofluorescent methanogens.

3.2 Correlations between microbial population using by different quantification techniques

The relationship between the distinct quantification techniques for the assay and each stage was studied by Pearson bivariate correlation. In the cases where Pearson´s correlation coefficient is higher than 0.8, it is studied if the relationship is lineal using the method of linear regression. The R2 coefficients were shown in the table 2.

Table 2 Linear regression coefficient between the distinct quantification techniques.

OLR ($gVST\ L^{-1}\ d^{-1}$)	TECHNIQUES	DAPI	AUTOFLUORESCENCE	EUB338	ARC915	MB1174	MX825
4.42	DAPI					0.735	
5.07	DAPI				0.676		0.773
	ARC915					0.757	0.968
	MB1174						0.698
5.92	DAPI			0.793			
7.50	DAPI			0.910	0.920		0.810
	EUB338				0.885		0.884
	ARC915						0.877

In the starting-up, the DAPI technique is correlated to the FISH technique using by MB1174 probe. The relation among the hydrogen generated in the hydrolysis and its consumption by the H_2-utilising methanogens, they would justify the linear correlation between these techniques, since the most of total population is hydrolytic during starting-up.

In the next stage, the DAPI technique is correlated to the FISH technique using by ARC915 and MX825 probes; ARC915 probe to MB1174 and MX825 probes; MB1174 to MX825 (see table 2). FISH technique using by MB1174 probe presents an inverse correlation with the others due to the beginning of acetoclastic methanogenesis. These results are in agreement with the status of functioning of the digester. The methane yield coefficient increases from 0.02 to 0.43 $LCH_4\ gCODd^{-1}$ and its percentage in the biogas from 11 to 25 %. The methane yield coefficient is superior to the maximum calculated theorist, since it starts the hydrolysis and fermentation of accumulated wastes during the first stage. This is reflected in a considerable increase of the *Archaea* from $0.14x10^9$ to $0.79x10^9$ cell $gVTS^{-1}$ and in particular of the acetate-utilising methanogens from $0.04x10^9$ to $0.82x10^9$ cell $gVTS^{-1}$ (see table 1).

In the third period, organic loading rate increases from 5.07 to 5.92 $gVTS\ L^{-1}d^{-1}$, causing the reactor is destabilized. Since the size of methanogenic population is not enough to degrade such quantity of organic matter. The acetic acid begins to accumulate, increasing the total acidity from 5812.82 to 6837.13 $mg\ L^{-1}$. The biogas production rate decrease from 1.93 to 1.15 $L_{biogas}\ L_{reactor}^{-1}d^{-1}$ and the methane yield coefficient from 0.43 to 0.34 $LCH_4\ gCODd^{-1}$. So, the organic matter in this stage was destined to the increase of the size of population, instead of the biogas production. *Archaea* increases from $0.79x10^9$ to $0.96x10^9$ cell $gVTS^{-1}$, while *Eubacteria* is maintained constant (see table 1). In this stage, the hydrogen has disappeared, favouring the presence of acetate-utilising methanogens and, therefore, the increment of the methane in the composition of the biogas from 25 to 50 %. For that reason, the DAPI technique is correlated to FISH technique using by EUB338 (see table 2).

In the latter period, organic loading rate increases from 5.92 to 7.50 $gVTS\ L^{-1}d^{-1}$. In this stage, it is observed like the principal physical-chemical parameters are maintained constants. Nevertheless, the concentration of

microbial population increases, in spite of the fact that the velocity of dilution is bigger. This phenomenon is due to the increment of organic loading rate that produces a great availability of the substratums. Therefore, in this period the DAPI technique is correlated to FISH technique using by EUB338, ARC915 and MX825 probes. In like manner, EUB338 probe is correlated to ARC915 and MX825 probes; ARC915 probe to MX825 probe (see table 2). This increment generalized in the coefficient of linear correlation is due to the stabilization of the size of the population that is produced when the reactor operates in stable conditions.

Finally, if it is studied the Pearson's correlation among the quantification technique for the assay completed is observed that: EUB338 probe is correlated to ARC915 and MX825 probes; ARC915 probe to MX825 probe, obtaining R^2 linear regression coefficient of 0.784, 0.782 and 0.936, respectively.

4. Conclusions

It can be concluded that DAPI technique was representative of principal microbial groups contained in the digester in each organic loading rate studied since it was related to FISH technique using MB1174 probe during starting-up and to EUB338, ARC915 and MX825 probes during stabilization. However, the lack of any apparent correlation between autoflurescence microscopy and FISH technique using by ARC915, MB1174 and MX825 probes could be must that this quantification technique was not representative of the methanogenic population. It allows only the quantification of the methanogens that present a high content of cofactor F_{420} such as H_2-utilising methanogens [4]. Nevertheless, the results obtained by MB1174 probe are two orders of magnitude higher than those obtained by autofluorescence microscopy. It could be possible that the percentage of autofluorescent H_2-utilising methanogens is very low. FISH technique was necessary to carry out the determination of main microbial population, in particular the methanogenic.

Acknowledgements The support by the Spanish Ministry of Science and Technology and Education and Science, projects PPQ2001-4032 and CMT2004-01655 is gratefully acknowledged. B.Montero thanks funding from Council of Education and Science of Junta de Andalucia. The authors thank Dr. T. Curtis and Dr. R. Davenport (Department of Civil Engineering and Geosciences. Newcastle University) for teaching molecular techniques using in this work.

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Comparison of different analytical processes for patulin determination in apple juice

F. M. Valle-Algarra[1], R. Mateo[1], Á. Medina[2], F. Mateo[3], E. M. Mateo[2], E. Sanchis Blanco[2], J.V. Gimeno-Adelantado[1], J. Peris-Vicente[1] and M. Jiménez[*,2]

[1] Dpto. de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjassot, Valencia, Spain
[2] Dpto. de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjassot, Valencia, Spain
[3] Dpto. de Ingeniería Electrónica, Universidad Politécnica de Valencia, Camino de Vera 14, E-46022, Valencia, Spain

[*]Corresponding author: e-mail: misericordia.jimenez@uv.es, Phone: +34 963543144

Three methods for determination of patulin in apple juice have been studied comparatively. The selected procedure for liquid samples implies liquid-liquid extraction with ethyl acetate/hexane (96:4, v/v). The collected patulin was then analyzed by liquid chromatography with UV-Visible detection. The obtained recovery was 82.9±3.2 % and the limit of detection was 2.0 µg/kg of juice, which are according to the valid guidelines established by the European Union. The developed analytical method has been used for the determination of patulin in various Spanish apple juices, where this mycotoxin has not been detected.

Keywords Patulin, apple juice, analytical process, *Penicillium expansum*

1. Introduction

Patulin (PAT) {4-hydroxy-4H-furo[3,2-c]piran-2(6H)-one} is a hemiacetalic lactone mycotoxin produced by certain species in the genera *Aspergillus, Byssochlamys* and *Penicillium*, mainly by *Penicillium expansum* [1,2]. PAT is a low-weighted molecule stable in aqueous acid media and unstable at basic pH [1], and at high temperatures [3].

This mycotoxin may be found in fruits, especially in apples and apple-derived products, where is currently the most important contamination source [4]. PAT shows a wide variety of toxicity effects in humans, as nausea, vomits and gastrointestinal disorders [5] and is considered immunotoxic, genotoxic, embriotoxic and neurotoxic [6], but no appropriate evidence exists for carcinogenicity in experimental animals. Therefore, the Cancer Research International Institute has included it in the Group 3, as there are no enough data about PAT effect in humans for its classification [2].

PAT remaining in commercial apple juice after the industrial processing for apple juice concentration is considered as an alimentary safety concern [7] due to its toxicity, especially for infants. The provisional maximum tolerable daily intake (PMTDI) has been established to 400 ng/kg body weight by day (safety factor 100) by the JECFA and, recently, by the European Union Food Scientific Committee (SCF) [8,9].

PAT is considered a quality marker of the apples entering the processing factory and levels in the final product are subject to regulatory control. The European Commission established maximum permitted levels of PAT in fruity foodstuffs (50 µg/kg for apple fruit juice and 10 µg/kg for apple products intended for infants and young children) [10]. Nevertheless, the new regulation introduces a lower limit in food intended for young children and infants, which show a higher consumption of fruit products [11].

Some methods have been developed for measuring PAT in apple juice concentrate. Among them, liquid chromatography (LC) coupled with UV detection is particularly well suited [12]. Methods such as multi-step liquid–liquid extraction with ethyl acetate and purification by Na_2CO_3 solution have been widely used as sample pretreatment [1,7,10,13]. However, liquid–liquid extraction is expensive and time-consuming, owing to the use of large amounts of organic solvents, which involve environmental problems and Na_2CO_3 clean-up can deactivate PAT [14]. In recent years, new procedures of extraction and clean-up using solid-phase extraction (SPE) have been validated [12,13,15,16]. These procedures are reportedly more reliable and repeatable with increased throughput, better recoveries and higher sensitivity, enabling the determination of much lower toxin levels [17]. The method adopted by the European Committee for Standardization as European Standard for determination of PAT in clear and cloudy apple juice is a LC method that has been validated by a collaborative study of PAT levels of 10 µg/kg or higher [18]. This method uses ethyl acetate and silica SPE column for extraction and clean-up, respectively.

The aim of this work was to compare the analytical processes of extraction and clean-up for PAT determination by Iha *et al.* [1] and MacDonald *et al.* [19] methods. They were also modified to optimize the methodology in order to improve the sensitivity and reproducibility, using LC-UV.

2. Experimental

2.1 Samples

A set of 56 apple juices, containing as a main component apple and/or pear, were purchased in Spanish supermarket and stores. These samples were stoked in their original package at 5ºC, and were open the same day of the analysis.

2.2 Chemicals and reagents

The following standards were purchased for the analysis: PAT, 5-hydroxymethylfurfural (HMF), dihydrated monosodium phosphate and sodium bicarbonate (Sigma-Aldrich, Alcobendas, Spain), anhydrous sodium carbonate, anhydrous sodium sulphate, sodium acetate and acetone (Panreac, Barcelona, Spain), acetonitrile, methanol, hexane, ethyl acetate, acetic acid, SPE-silica cartridges (J.T. Baker, Deventer, The Netherlands). All solvents were LC grade. SPE C18 cartridges (Waters, Milford, MA, USA), filter paper (Whatman No 4, Whatman, Maidstone, UK) were used as well. Pure water was generated in-situ by a Milli-Q pure water production device (Millipore, Milford, MA, USA).

The acetic acid/acetate buffer solution (AABS) was prepared by addition of 0.45 ml of acetic acid and 0.15 g of sodium acetate to 40 ml of water, then adjusted to pH 4 with acetic acid. The volume was finally brought to 50 ml.

A 10-ml stock solution PAT (1.0 mg/ml) in ethyl acetate was prepared. This solution was stored at –20ºC. Working standards were prepared by evaporation of the appropriate volume of the stock solution with N_2 at room temperature. Furthermore, they were solved in the corresponding volume of AABS. The calibration curve was prepared by LC analysis of these solutions.

2.3 Extraction and purification

The extraction and purification methods described below (named by their developers) were applied to previously analyzed free-PAT apple sample. The samples were spiked to reach concentrations of 0.1 or 1.0 mg of PAT/kg in each sample.

2.3.1 MacDonald procedure

A 10-g sample of cleaned juice and 20 ml of ethyl acetate was introduced in a 250-ml separatory funnel and the mixture was shaken for 1 min. The extraction was repeated three times and the three organic phases obtained were mixed in another separatory funnel. Four ml of 1.5% aqueous Na_2CO_3 was added and the mixture was shaken for 30 s, and then the organic phase was filtered through paper filter containing 15 g of anhydrous Na_2SO_4. The extraction procedure was repeated three times and the obtained solution was evaporated under a N_2 stream at room temperature. The remaining solid was solved in 1 ml of AABS solution and a 50-µl aliquot was introduced in the liquid chromatograph using the AOAC Official Method 2000.02 [19].

2.3.2 Iha procedure

Apple juice was homogenized and 5 g was introduced in a 50-ml centrifuge tube, together with 0.5 g of sodium bicarbonate and 5 ml of ethyl acetate/hexane (96:4, v/v). The sample was shaken for 190 min in an orbital shaker, and then centrifuged for 3 min at 3000 rpm. A volume of 3 ml of the organic phase was transferred to glass tube, together with 30 µl of acetic acid, and then the solvent was evaporated to dryness under N_2 stream at room temperature. Immediately, 1 ml of AABS was added, and the mixture was vigorously vortexed for 1 min [1].

2.3.3 Li procedure

C_{18} SPE cartridges were preconditioned with: i) 10 ml of methanol, ii) 3 ml of 10% methanol in aqueous solution and iii) 10 ml water. The cartridges were not allowed to dry. One gram of sample with 0.5 ml AABS

solution was transferred to SPE cartridge and solution sample was pushed at 2-3 ml/min with a plunger. Then, 5 ml of hexane was added and the column was dried with air stream for 15 min. This eluate was discarded and then the cartridge was sequentially eluted with 5 ml hexane/ethyl acetate/acetone (1:5:4), 5 ml of hexane/ethyl acetate/acetone (1:4:5) and 5 ml of hexane/ethyl acetate/acetone (1:3:6) in this order. Eluates were combined, acidified with 30 µl of glacial acetic acid and the solution was dried with nitrogen stream at 30 ºC. Dry extract was immediately solved in 1 ml AABS [15].

2.4 PAT determination by liquid chromatography

The LC system consisted of a Waters 600E quaternary pump, with a Waters 717 autosampler and a Waters 996 UV-visible diode array detector. Millennium 32 (3.05.01) was used as the software to control the system and to process the obtained signals (Waters, Milford, MA, USA). The separation was performed at constant temperature (30ºC) in a C18 reversed-phase column (Agilent Zorbax XDB-C18® 150 mm length; 4.6 mm i.d., 5 µm particle size) connected to an Agilent Zorbax guard column (4 mm length; 3 mm i.d.; 5µm particle size) filled with the same stationary phase. Elution was carried out with a mixture of acetonitrile (solvent A) and water (solvent B). The following gradient program was applied: i) solvent B 99% (maintained for 2 min); ii) lineal change of B from 99% to 90% in 11 min; iii) lineal change of B from 90% to 1% in 3 min; iv) 1% B during 1 min; v) return to the initial conditions in 1 min. Detection wavelength was fixed at 276 nm and injection volume was 50 µL.

3. Results and discussion

3.1 Comparison of the analytical procedures for apple juices

The studied methodologies were an official method [19], and two methods [1,15] considered easier than the official one to improve the recovery, selectivity and detection limits (Table 1).

The highest recoveries for apple juice samples spiked with 1 mg PAT/kg were obtained by the MacDonald and Iha methods (81.0% and 72.1%, respectively). However, the relative standard deviation (RSD) of MacDonald method (12.3%) was too high. Moreover, the limit of detection (LOD) is not too high, considering that a 10:1-preconcentration has been made, probably increasing also the amount of the impurities. The MacDonald method is both time and organic solvent consuming, rising cost and being environmentally unfriendly (Section 2.3.1). On the contrary, the Iha method is fast, easy, reproducible and shows low LOD (2.1 µg/kg). Thus, it can be considered as the best of the studied methods.

Table 1. Recovery, relative standard deviation (RSD) and limit of detection (LOD) values for PAT in apple juices spiked with 0.1 and 1.0 mg of mycotoxin/kg by the studied procedures.

Procedure	Spiked PAT (mg/kg)	Recovery (%)	RSD (%)	LOD (µg/kg)
Iha [1]	0.1	74.3	1.7	2.1
	1.0	72.1	3.1	
MacDonald [19]	0.1	73.2	8.5	1.8
	1.0	81.0	12.3	
Li [15]	0.1	68.3	9.9	6.6
	1.0	72.5	11.0	

Values are averages of three determinations.
LOD was calculated as three times the standard deviation of the blank, and LOQ was three times LOD [20].

At a spiking level of 0.1 mg of PAT/kg sample, the recovery using the Li method (68.3%,) was lower than that using the Iha method (74.3%). RSD of the first method was high (9.9%), which indicates low precision; moreover, its LOD was the highest (6.6 µg/kg).

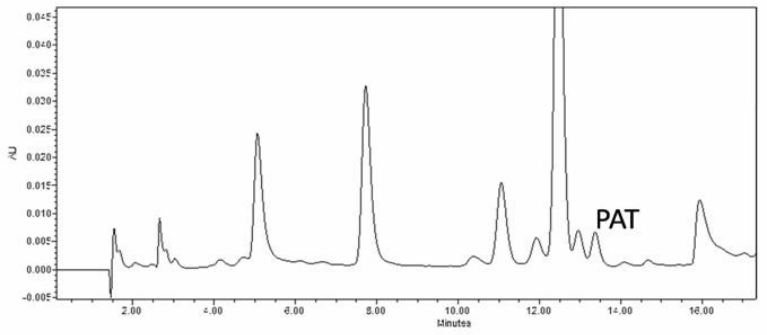

Fig. 1. Chromatogram obtained from the analysis of apple juice spiked with 0.1 µg PAT/ml using the Iha procedure. Detection wavelength was 276 mn.

In order to improve the Iha method, several proportions of an ethyl acetate-hexane solvent mixture were tested. The highest recovery (73.4%) was found when the ratio was 96:4 v/v.

Fig. 1 shows a chromatogram obtained from an apple juice sample spiked with PAT 0.1 mg/kg analyzed by the Iha procedure.

3.2 Occurrence of PAT in apple samples

Samples of apple juice were analyzed by Iha procedure. The whole set of samples contained PAT below the limit of quantification (LOQ = 6.3 µg/kg), although the mycotoxin was detected (LOD = 2.1 µg/kg) in four samples. Therefore, all the analyzed samples contained values below the maximum allowable limits fixed by the European Union.

4. Conclusions

After the comparison of the methods for the determination of PAT in apple juices, the Iha method has been proposed as the best procedure because it is simple and fast, implies a simple liquid-liquid extraction, and the LOD is low enough to be applied to detect samples surpassing the maximum levels for PAT established in the European Union Regulation.

Acknowledgements The authors wish to thank financial support from the Spanish Government "Ministerio de Educación y Ciencia" (Projects AGL-2004-07549-C05-02/ALI, and for two research grants) and from the Valencian Government (Project GV04B-111).

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Electroimmunoassay for detection of bacterial cells

M. Hrubeš[1], **L. Červenka**[1], **J. Vytřasová**[2], **I. Pešková**[1], and **K. Vytřas**[*, 1]

[1] Deparment of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, nam. Čs. Legií 565, 53210 Pardubice, Czech Republic

[2] Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubnice, Štrossova 239, 53003 Pardubice, Czech Republic

[*]Corresponding author: e-mail: karel.vytras@upce.cz, Phone: +420 466037512, Fax: +420 466037068

An amperometric method for *Salmonella* detection is presented as an example of electroimmunoassay of bacterial cells. The method is based on the reaction of salmonella with an enzyme-linked specific antibody (alkaline phosphatase), forming salmonella-antibody-alkaline phosphatase (SAAP) conjugates. After their hydrolysis, the products formed (phenols) are detected amperometrically using a carbon paste electrode; current signals were monitored at 0.65 V *vs.* Ag/AgCl/sat. KCl. A medium of pH = 10.00 was found as the best. Amount of phenol generated by SAAP was proportional to the number of *Salmonella* Enteritidis in a sample.

Keywords Salmonella Enteritidis; carbon paste electrode; amperometric detection; alkaline phosphatase

1. Introduction

Evaluation of food quality is connected with numerous questions. One of them is undesirable occurrence of pathogenic microorganisms, which are dangerous to human health. Their detection, usually based on classical cultivation methods, is tedious and, on the other hand, financially and materially demanding. For those reasons, a search for new methods which are rapid and applicable for wider spectrum of microbes is very important and actual. One of the perspective solutions can be based on applications of electroanalytical methods combined with procedures used in microbiology and immunology. Voltammetric, impedimetric or amperometric methods have been used in this field. Amperometric methods for the determination of bacterial biomass are based on measuring the current that is produced at a working electrode poised at a constant potential [1,2]. The measurement can be conducted with two- or three-electrode systems [2]. Specific anti-pathogen antibodies that are often used can be immobilized [3, 4] or they can simply be diluted in the antigen (a pathogenic microorganism to be determined) solution [5]. During the incubation, a conjugate between the pathogen and specific antibodies is formed. The use of enzyme-linked specific antibodies has great advantage. In the most cases, they are linked to alkaline phosphatase [3-7], but horseradish peroxidase [8, 9] or ß-galactosidase [10] could also be used as well. The enzyme choice has an influence on the substrate selection that is utilized by the enzyme; 1-naphthyl phosphate [3, 4, 6] or phenyl phosphate [5] are usually applied. Alkaline phosphatase hydrolyses the substrate and the product formed is detected by amperometric measurement. The resulting amount of the product is proportional to the number of bacterial cells in the sample.

As an example, a method development is presented and discussed dealing with determination of *Salmonella* Enteritidis based on amperometric detection of phenol deliberated in the course of the biocatalytic reaction mentioned above.

2. Experimental

2.1 Preparation of composite materials

Mixture of Carbon CR5 powder 5.5-7.0 μm in diameter (Maziva Týn n. V., Czech Republic) and mineral oil M5904 (Sigma Aldrich, Germany) in different ratios have been prepared. The homogenic mixtures were packed in teflon piston holder developed by Švancara and co-workers [11]. The working diameter of CPE was 2 mm.

2.2 Voltammetric measurements

Three electrode system consisting of CPE (working), Ag/AgCl/saturated KCl (reference) and platinum wire (counter electrode) connected to PalmSens (Ivium Technologies, Netherland) was used for cyclic voltammetry (scan rate 100 mV/s, range of potentials -1.0; +1.0 V). A Tris buffer solution (TBS) composed of 25 mM

tris(hydroxymethyl)aminomethan and 150 mM NaCl) with pH ranged from 2.77 to 11.50 with addition of 0.2 mM of phenol and with or without addition of 1.5 mM $MgCl_2$ was measured by cyclic voltammetry.

2.3 Characterization of composite material

The CPE´s prepared were characterized on the basis of its resistance and cyclic voltammograms in both TBS solution (pH 10) and redox system (1M KCl and $2x10^{-4}$ M $K_3Fe(CN)_6$). The CPE with the best characteristics was selected for further experiments.

2.4 Amperometric measurements

All the amperometric measurements were carried out in three-electrode system as described above and the effect of pH, potential and steering on the current connected with phenol oxidation was evaluated. The following ranges were examined: TBS solution at pH´s ranges from 2.77 to 11.50, the range of potentials from 0.4 to 0.9 V and the steering from 100 to 700 rpm. All the measurements were conducted with addition of 24.4 μl phenol in 4 ml of working solution.

2.5 Sample preparation for amperometric measurements

The Yang´s et al. technique [5] was adopted and modified in this study. The bacterial culture of *Samonella* Enteritidis (Egg yolk isolate, KBBV, University of Pardubice) was prepared by cultivation in Brain Heart Infusion broth (BHI, HiMedia, India) at 37°C for approximately 24 h to reach the final density of the bacterial cells corresponding to the 4th degree of the McFarland turbidity standard (ca. $12x10^8$ CFU/ml). A volume of 5 ml of freshly grown culture was heated at 90°C for 15 min. The heat-killed culture was serially diluted in TBS buffer (pH 7.40) containing 0.1 % Tween-20. A portion (1 ml) of each dilution was added to microtube and was incubated with alkaline phosphatase-linked antibodies to *Salmonella* antigens in a 0.2 ng/ml (CSA-1, Kirkegaard & Perry Laboratories Inc., USA), 0.1% Tween-20 in TBS buffer solution (pH 7.40) at 37°C for up to 3 h. During the incubation period, salmonella-antibody-alkaline phosphatase conjugate (SAAP) was formed. Thereafter, the sample was filtered using polycarbonate membrane (0.20 μm, Filalbet, Spain) to remove non-conjugated specific antibodies. The filtration membranes were washed three times using TBS buffer of the optimum pH determined in previous experiment. The SAAP conjugate attached on the surface of polycarbonate membrane was aseptically transferred into microtube containing TBS buffer (0.9 ml) and 1 mM phenyl phosphate. The solution was incubated at 37°C from up to 150 min. During incubation, phenyl phosphate was hydrolyzed by alkaline phosphatase forming phenol, an electroactive molecule which was then detected amperometrically using a procedure descibed above. The hydrolysis was stopped by addition of 0.1 M Na_2HPO_4 solution (0.1 ml). The incubation times of *S.* Enteritidis with enzyme-linked antibodies and the SAAP conjugate with phenyl phosphate were evaluated to give the highest signal of the phenol oxidation current.

2.6 Amperometric detection of *S*. Enteritidis

Measurements were performed using a three-electrode system described above. A volume of 1 ml of SAAP solution was added to 4 ml of TBS solution (optimum pH). The phenol amount was amperometrically determined under conditions resulting from previous experiments. After each individual measurement, a CPE surface was renewed by pushing 2 mm of the paste away and polishing its surface using a clean wet paper.

3. Results and discussion

Various ratios of carbon powder and mineral oil have been tested. The mixture of the CR5 carbon powder (0.5 g) and mineral oil (150 μl) was selected for further measurements in regard of its consistency, resistancy (9.4 ± 0.5 Ω) and potential range (-0.38 to 0.91 V). The background signal of bare CPE in TBS buffer at pH 10 was determined as 3 μA and the difference between anodic (E = 0.30 ± 0.02 V) and catodic current (E = 0.21 ± 0.02 V) of the reversible system of ferro- and ferricyanide resulted in 100-120 mV (Fig 1). The effect of the CPE composition on the phenol oxidation current was evaluated in TBS buffer (10.00 pH).

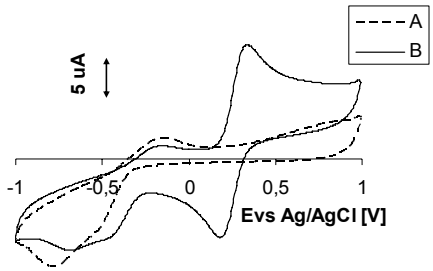

Fig. 1 The cyclic voltammogram of bare CPE in TBS at pH 10 (A) and in the reversible system of $K_3Fe(CN)_6$ / $K_2[Fe(CN)_6$ (B). Anodic current of oxygen (E = -0.18V), anodic current of ferricyanide E = (0.30 ± 0.02) V, catodic current of ferrocyanide E = (0.21 ± 0.02) V. Scan rate 100 mV/s.

The cyclic voltammetry of phenol (0.2 mM) in different TBS buffers showed the highest phenol oxidation current at pH 9.40-10.00, polarization potential being between 0.5-0.6 V. The addition of 1.5 mM of $MgCl_2$ as an activator made the peak of the phenol oxidation sharp and clearly distinguishable from the background signal.

Determination of phenol using amperometric detection strongly depended on working potential, pH of working solution (TBS buffer) and the rotation speed of stirrer. As was shown in Fig. 2a, the current of phenol oxidation occurred at + 0.55 V in a pH 10.00 buffer with addition of activator. However, this potential did not ensure a transit of limited current of phenol using CPE. The effect of various potential on the oxidation current of phenol is depicted in Fig. 2b. It is evident that the limited oxidation current was reached when +0.60 V was applied. For subsequent studies, a potential of +0.65 was chosen for phenol oxidation. Fig. 2c shows that the response of the CPE increased with the increase in the rotation speed from 100 to 500 rpm for 24.4 µM phenol and thereafter the magnetic stirrer tends to be instable and may damages the surface of the reference electrode.

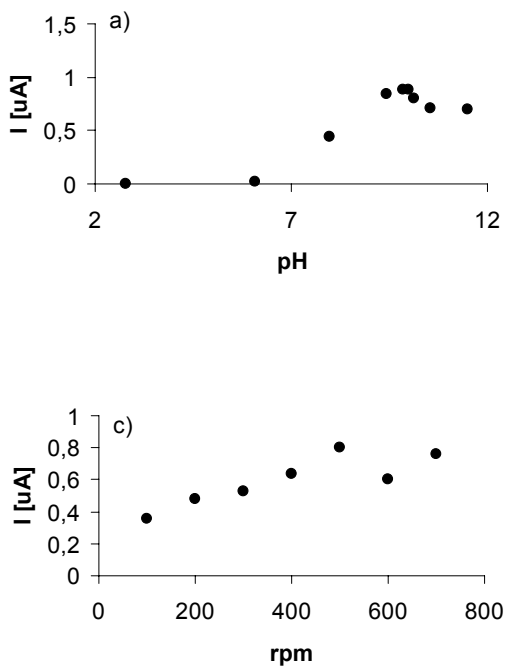

Fig. 2 Effect of pH of TBS buffer on the oxidation current of phenol (24.4 µM) at an applied potential of 0.65 V vs. Ag/AgCl reference electrode and 500 rpm of steering (a). Effect of working potential on the oxidation current of phenol (24.4 µM) in TBS (pH 10.00) and 500 rpm of steering (b). Effect of rotation speed of stirrer on the oxidation current of phenol (24.4 µM) in TBS (pH 10) at an applied potential of 0.65 V vs. Ag/AgCl reference electrode (c).

Rotation speed of 500 rpm was used in this study. Similarly to those obtained by cyclic voltammetry, amperometric measurement also showed that increase in pH of buffer solution resulted in the increase response of CPE to phenol. Hence the pH of TBS buffer was maintained between 9.8-10.0 with addition of 1.5 mM of $MgCl_2$.

The response of the CPE was linear in TBS (pH 10.00) from 2 to 10 mM phenol with a correlation coefficient of 0.998. Background signal of a negative control (TBS, pH 10.00) is 40 nA. The addition of 12.5 µl of 1mM phenol increased the oxidation current about 0.1 µA.

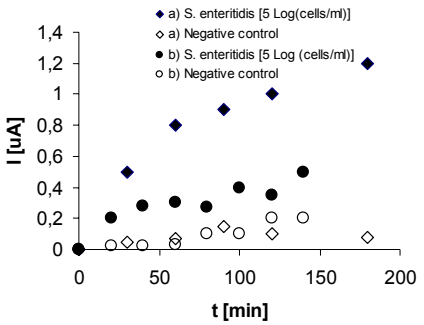

Fig. 3 The effect of incubation time of a) S. Enteritidis with enzyme-linked antibodies (0.2 ng/µl) in TBS buffer (pH 7.4) + 0.1% Tween-20 at 37°C and b) immunocomplex with 1mM substrate (phenylphosphate) + 1mg/ml MgCl2 in TBS (pH 10) at 37°C on the oxidation current of phenol. Working potential +0.65 V vs. Ag/AgCl reference electrode, working solution TBS (pH 10), rotation speed 500 rpm.

The oxidation current of phenol increased with the increase of incubation time of *S. E*nteritidis in the presence of alkaline phosphatase-linked antibody (Fig. 3). However, no current maximum was observed during 180 min incubation at 37°C. This procedure needs further investigation; nevertheless we used 30 min incubation period [5]. The current of phenol increased with the increase of incubation time of SAAP in the presence of 1 mM phenylphosphate (Fig. 3.). However, the current peaks of negative control (i.e. without salmonellas) have significantly increased after 60 min of incubation; therefore the incubation time of 60 min was proposed for releasing phenol.

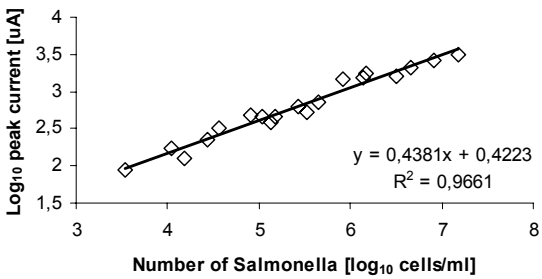

Fig. 4 The final peak current vs. concentration of the *Salmonella* Enteritidis sample solution in the electroimmunoassay.

After the operating conditions of the immunoelectrochemical assay was evaluated, a detection limit for *S.* Enteritidis was calculated to be $8.5x10^3$ cells/ml with an intercept of +0.42, a slope of 0.43 and a correlation coefficient of 0.983 (Fig. 4). Relative standard deviation of the assay was less than 10%. However, it should be noted that the current of phenol oxidation corresponded to 10^4 cells/ml occasionally gave the signal close to the negative control. Nevertheless, the results of this study showed a great potential of this method to be used as a rapid and sensitive method for detection of bacterial cells.

Acknowledgements The supports by Ministry of Education, Youth and Sports of the Czech Republic under projects LC366035 and MSM0021627502) and University of Pardubice, PRA/2007/12 are gratefully acknowledged.

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Fine structure of wild type and mit$^-$ mutants of the yeast *Saccharomyces cerevisiae*

Ezzatollah Keyhani[*]

Laboratory for Life Sciences, Saadat Abade, Sarve Sharghi 58, 19979 Tehran, Iran.

[*]Corresponding author: e-mail: keyhanius2002@yahoo.com

In this work, the morphology of wild type *Saccharomyces cerevisiae*, as well as that of mutants lacking subunit I, II or III of cytochrome *c* oxidase (OXI mutants), or lacking cytochrome *b* (COB mutant), was examined by electron microscopy. The strains were also characterized by their reduced-minus-oxidized difference spectra and oxygen consumption. The reduced-minus-oxidized difference spectra of intact cells showed the presence of cytochromes *c*, *b*, and *c* oxidase in the wild type, but no cytochrome *c* oxidase was detectable in any of the OXI mutants, while the COB mutant lacked cytochrome *b*. Oxygen consumption measurements showed that, except with wild type and COB mutant, the respiration was cyanide insensitive. Electron microscopy examination of permanganate-fixed *S. cerevisiae* showed that the most striking difference between wild type and mutants was alteration in mitochondrial form and, in some mutants, extensive abnormal form of mitochondria with longitudinal and sometimes circular cristae.

Keywords yeast OXI and COB mutants; absorption spectra; oxygen consumption; electron microscopy; Saccharomyces cerevisiae

1. Introduction

Mitochondria are energy-producing organelles, present with rare exception in all aerobic eukaryotic cells. They are semi-autonomous organelles with their own, small DNA that codes for 11 subunits of mitochondrial proteins. The large majority of mitochondrial proteins are coded by nuclear DNA, translated into the cytoplasmic ribosomes and transported into the mitochondria. Besides energy production, mitochondria play a key role in various cellular metabolisms, including Ca^{2+} regulation. More recently their role in apoptosis has been under intense investigation.

An important discovery in mitochondrial genetic and molecular biology was that of mit$^-$ mutants in the yeast *Saccharomyces cerevisiae* [1-3]. These mutants were characterized by a specific absence in mitochondrial components, among them cytochrome *c* oxidase and cytochrome *b*. Furthermore, it was shown by genetic analysis that mutation in specific loci on yeast mitochondrial DNA (mDNA) caused a selective loss of functional cytochrome *c* oxidase. These loci termed OXI I, OXI II and OXI III [4] corresponded to mutations affecting, respectively, subunit II, subunit III and subunit I of cytochrome *c* oxidase [2, 5]. In addition, a mutation in the locus termed COB prevented the formation of functional cytochrome *b*.

2. Materials and Methods

2.1 Strains and culture conditions

Yeast cells, *Saccharomyces cerevisiae*, were grown in synthetic medium with 100 μg copper per liter and 3% glucose as substrate, as described in reference [3]. Cells were harvested after 16 h culture and washed twice in 0.01 M phosphate buffer, pH 7.4, for further studies. *S. cerevisiae* strain 777-3C and detailed obtainment of OXI and COB mutants were described amply in reference [3].

2.2 Absorption spectra and oxygen consumption measurement

The room temperature reduced-minus-oxidized difference spectra of *S. cerevisiae* cells (wild type and mutants) were recorded with an Aminco-DW2 spectrophotometer and the oxygen consumption was measured with an oxygen electrode, as described in reference [6].

2.3 Thin sections and electron microscopy

A small pellet of yeast cells was fixed in 5% potassium permanganate at room temperature (24°C) for 2 hours and embedded in epoxy resin as described in reference [6]. The thin sections obtained were examined in a Philips EM 300 electron microscope, calibrated by a carbon grating replica, at an accelerating voltage of 80 kV with a 30-μm objective aperture.

3. Results

3.1 Reduced-minus-oxidized difference spectra and oxygen consumption

Figure 1 shows the room temperature dithionite-reduced-minus-H_2O_2-oxidized difference spectra of wild type and selected mutants. In the wild type, the α absorption band due to cytochrome *c* oxidase was seen at 605 nm, while cytochromes *b* and *c* exhibited absorption bands at 560 and 550 nm respectively. In the COB mutant, the absorption band at 560 nm due to cytochrome *b* was undetectable while the absorbance bands due to cytochromes *c* oxidase and *c* were present. In all three OXI mutants, the absorption band due to cytochrome *c* oxidase was undetectable while those due to cytochromes *b* and *c* were present.

Figure 2 shows the oxygen consumption of wild type and OXI II mutant. As seen in Fig. 2, the wild type showed a good endogenous respiration which was enhanced by ethanol and glucose and was inhibited by cyanide; on the other hand with the OXI II mutant, respiration was detected only upon addition of ethanol and it was insensitive to cyanide. Results were similar with the other OXI mutants. In COB mutant, however, respiration was detected upon addition of either glucose or ethanol as in the wild type, although no endogenous respiration was detectable; unlike OXI mutants, COB mutant respiration was sensitive to cyanide.

3.2 Morphology: general features

S. cerevisiae wild type and mutants shared certain structures which were present in all of them: 100-200 nm cell wall, periplasmic space, 7 nm-thick membrane which was almost apposed to the cell wall, plasma membrane invagination towards the cytoplasm, nucleus, mitochondria, endoplasmic reticulum which in all cases was more elongated when close to the plasma membrane and shorter when scattered through the cytoplasm, Golgi apparatus, secretory vesicles, vacuoles of different sizes, and peroxisomes. These structures were similar to those present in higher eukaryotic cells. Cell size varied between 5-7 μm in length and 3-4 μm in width.

3.3 Specific wild type and mutants morphology

Wild type *S. cerevisiae* 777-3C is shown in Figure 3. The cells were generally round or oval (5-7 μm long; 3.5-4 μm wide), with a nucleus of irregular form; large vacuoles (of 1.5 μm diameter), highly electron-dense, were also visible. Mitochondria (of 0.7 μm long to 0.2 μm wide), circular or oval in section were scattered through the cytoplasm. Endoplasmic reticulum, variable in size (0.4-2 μm), was also scattered in the cytoplasm, being larger when located beneath the plasma membrane and smaller when scattered through the cytoplasm, in wild type as well as in mutants. Lysosomes (0.2 μm) were also seen.

The COB mutant (Fig. 4) also showed a nucleus irregular in shape but containing up to 13 pores in this section. A vacuole of 2 μm diameter, containing material dense to electrons (Fig. 4), abundant lysosomes, some of them with a tail of 0.4 μm (Fig. 8), and an autophagic vacuole (Fig. 8) were detectable. Mitochondria were small (0.2 to 0.75 μm in length) and scattered through the cytoplasm. At least 3 vacuoles without electron dense material were also seen (Fig. 4).

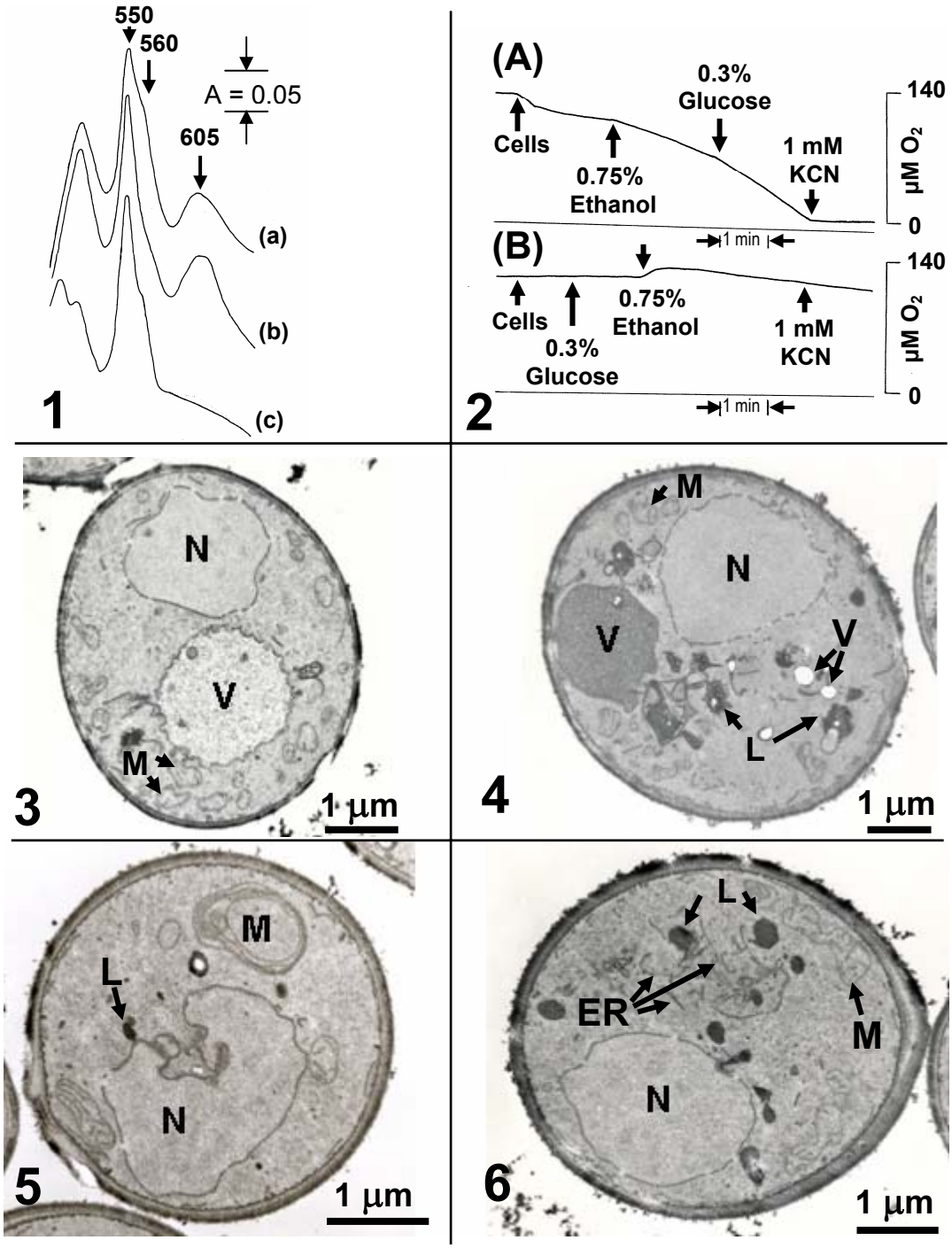

Fig. 1 Selected reduced-minus-oxidized difference spectra of wild type (a), and COB (b) and OXI II (c) mutants. **Fig. 2** Oxygen consumption of wild type (A) and OXI II mutant (B); 4 x 10^8 cells were used per assay. **Fig. 3** Wild type *S. cerevisiae* cell; **Fig. 4** COB mutant cell; **Fig. 5** OXI I mutant cell; **Fig. 6** OXI II mutant cell. N: nucleus, V: vacuole, M: mitochondria, L: lysosome, ER: endoplasmic reticulum. For details, see text.

As shown in Figure 5, OXI I mutant exhibited a highly irregular nucleus, rare mitochondria, some of them with rare circular cristae. The lysosomes (up to 0.2 µm in diameter) were scattered through the cytoplasm, some of them being very small (0.08 µm in diameter, the size of microsomes).

Figure 6 shows OXI II mutant with oval nucleus. Mitochondria with irregular contour were visible. Lysosomes (0.2-0.4 µm in diameter) and endoplasmic reticulum were abundant.

OXI III mutants exhibited an irregular nuclear shape (Fig. 7) but their most striking feature was the changes observed in inner mitochondrial membrane cristae. Sometimes the inner membrane cristae crisscrossed the mitochondria (Fig. 7), while in other instances the mitochondrial inner membrane was either circular (Fig. 12) or rather longitudinal (Fig. 13). Vacuoles as long as 2.2 μm, still containing organelles, were also seen (Fig. 9). Some cells exhibited abundant endoplasmic reticulum (Fig. 10), and others, a very active Golgi, with numerous vesicles (up to 0.5 μm long, Fig. 11).

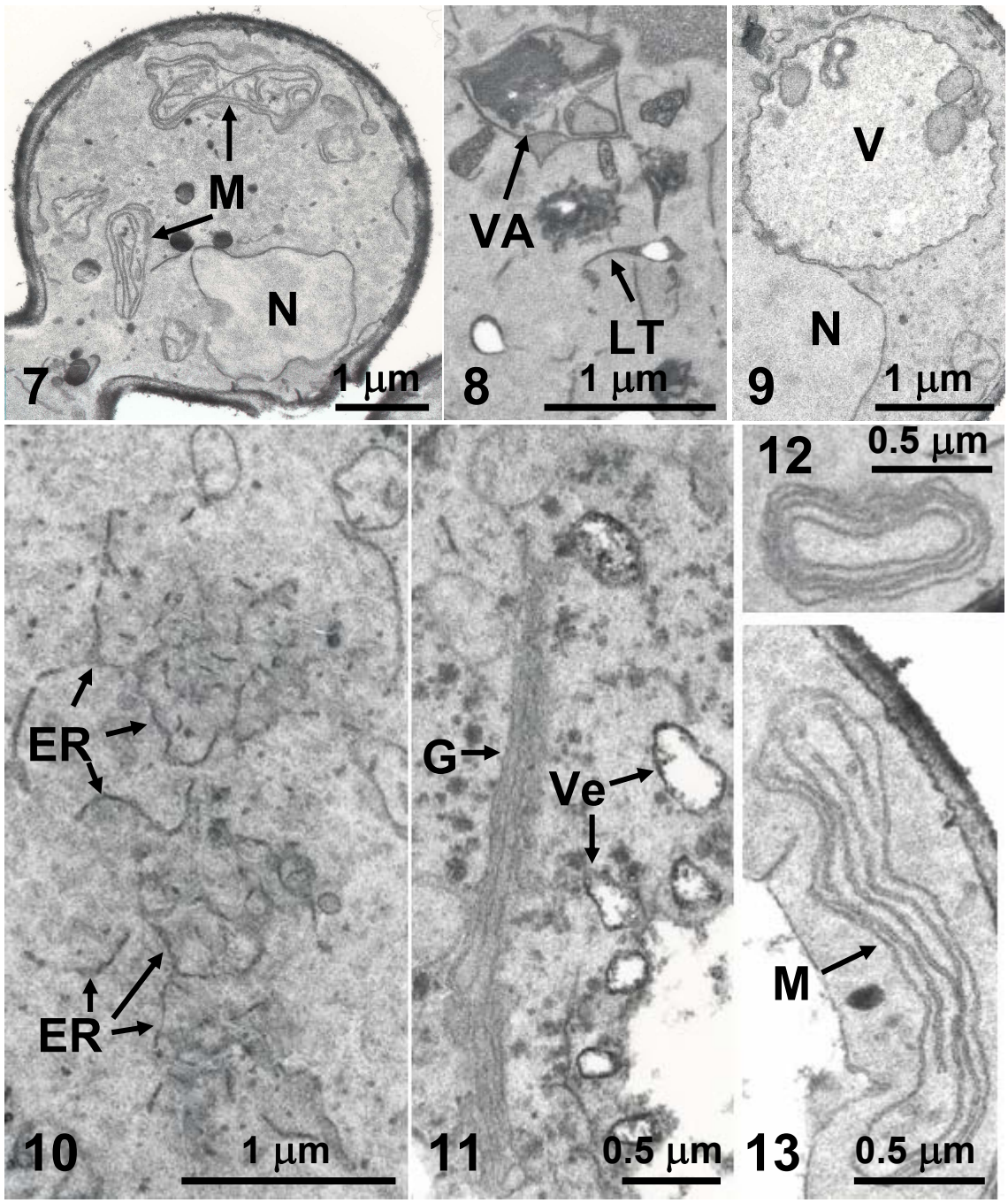

Fig. 7 OXI III mutant cell; note the abnormal shape of mitochondria. **Fig. 8** High magnification of a portion of Fig. 4 (COB mutant); note the abundance of lysosomes, the lysosome with tail (LT) and the autolytic vacuole (VA). **Fig. 9** Higher magnification of cytoplasmic region in OXI III mutant, with vacuole (V) containing four organelles. **Fig. 10** Formation of abundant endoplasmic reticulum (ER) in OXI III mutant. **Fig. 11** Golgi of OXI III mutant with abundant vesicles. **Fig. 12, 13** Selected mitochondria in OXI III mutant. N: nucleus, M: mitochondria, G: Golgi, Ve: vesicle.

4. Discussion

The OXI mutants all lacked functional cytochrome *c* oxidase, yet they did not resemble each other. They showed an extremely heterogeneous morphology of cytoplasmic organelles. The situation was identical with COB mutant. Some cells had mitochondria of reasonably normal aspect; others showed very small mitochondria with poorly developed cristae. Lysosomes were very abundant in some cells, very rare in others. This suggested that besides producing point mutations and specific deletion of cytochrome *c* oxidase and cytochrome *b* subunits, the mutagenesis did also affect other cellular metabolisms, either directly by defect in cellular respiration, or by multiple other mutations that altered different metabolic pathways. Mitochondria are known to form a reticulum in yeast cell [7]. However, cells containing multiple mitochondria independent of each other have also been seen in numerous eukaryotes and some authors suggested that mitochondria were morphologically and functionally heterogeneous within cells [8]. A number of genes have been shown to be involved in mitochondrial structure and function [9]. A total number of 341 ORFs were identified to be required for respiratory growth, 38 of which encoding unknown proteins [9]. It was also suggested that a total of 119 essential genes were required for the maintenance of mitochondrial morphology [10]. In the present work, the most extensive cristae changes were seen in OXI III mutants lacking subunit I of cytochrome *c* oxidase, suggesting that this subunit had a key role in the morphology of the inner mitochondrial membrane.

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Study of necessary pre-treatment for application of microbial techniques of quantification to high content in solids samples

B. Montero*, J.L. García-Morales, D. Sales, and **R. Solera**

Department of Chemical Engineering, Food and Environmental Technologies, Faculty of Marine and Environmental Sciences, University of Cadiz, Campus Rio San Pedro s/n, 11510 Puerto Real, Spain

*Corresponding author: e-mail: blanca.montero@uca.es, Phone: +34 956016157

The application of count techniques in thermophilic-dry anaerobic reactor of Organic Fraction of Municipal Solid Waste (OFMSW) requires an adequate pre-treatment due to high content in solids present in the effluent. Therefore, an experiment was designed to select the best conditions of pre-treatment. Four conditions were assayed: without pre-treatment, shaking (60 seconds), addition of tensioactive agent (20µL of Tween 80 with a concentration [5µg/µL]) and sonication (5 seconds). The technique of quantification used to determine the best pre-treatment was the DAPI epifluorescence microscopy method. All quantitative data were checked for normality before Paired-Samples T Test and an analysis of variance (ANOVA) with Spss v11.5 program. It can be concluded that the most appropriate pre-treatment applied for microbiological count of high solids content samples was the addition of Tween 80 and 120 seconds of shaking.

Keywords high content in solids, DAPI, pre-treatment, shaking, Tween 80, sonication

1. Introduction

Different procedures of microbial quantification by epifluorescence microscopy have been reported in the references [1, 2]. However, in the case of complex samples (extreme diversity of microorganisms) such as they are from biological treatment of wastes, fermentations or environmental samples, the procedure of microbial quantification employed was epifluorescence microscopy by fluorochrome or by in situ hybridization. The latter procedure is molecular, and therefore, high specificity. This technique results very attractive and it is been carried out as control of microorganisms in complex microbiological process.

Nevertheless, a lot of interferences were produced in the stage of visualization and count when the samples present high content in solids, making difficult or impossible to accomplish these stages.

Therefore, a previous stage was necessary to improve the conditions of the samples. There are distinct methods to improve the conditions of the samples before their count by microscope. However, there is not a common procedure, in spite of the results of microbial quantification depend on this previous stage.

The main objective of this work was to select the best conditions of pre-treatment. Four conditions were assayed: without pre-treatment, shaking (60 seconds), addition of tensioactive agent (20µL of Tween 80 with a concentration [5µg/µL]) and sonication (5 seconds). The technique of quantification used to determine the best pre-treatment was the DAPI epifluorescence microscopy method. All quantitative data were checked for normality before Paired-Samples T Test and an analysis of variance (ANOVA) with Spss v11.5 program.

2. Material and Methods

2.1 Collection of samples

Samples were collected from a thermophilic-dry anaerobic reactor. The equipment consisted of a laboratory-scale continuously stirred tank reactor (CSTR), with no recycling of biomass working in semicontinuous regime (one dose per day) in the thermophilic range (55ºC).

The reactor was loaded with 1.5 kg of milled dry synthetic OFMSW (90% TS). The moisture was adjusted using an inoculum from SEBAC (Sequential Batch Anaerobic Composting) that consisted of a 1:1 v/v mixture of thermophilic sludge and leachate [3-5].

A synthetic feed based on the nutritional requirements of the main populations of microorganisms involved was prepared [6]. This type of feed avoids the problem of high variations in the composition of the source selected OFMSW.

The reactor was operated from 40 to 25 days of retention time (RT). The organic loading rate added to the system was modified, but a constant organic loading rate was maintained in each RT. It was used four organic loading rates: 4.42, 5.07, 5.92 and 7.50 kg volatile solid m^{-3} day.

2.2 Quantification techniques

Quantification of total numbers contained in the system was determined by epifluorescence microscopy with DAPI, according to Kepner [7, 8]. Immediately after sampling, the sample was fixed with a glutaraldehyde solution buffer (final concentration, 5%). 10 mL of sample diluted using phosphate buffer (pH 7.2) to give a count of 10-15 cells per field and homogenised with a vortex Heidolph Reax 2000, for 30 s. Bacterial cells were retained on a 25 mm black polycarbonate membrane filter with a 0.22 µm pore size (Millipore GTBO). Cells were counted visually using a Nikon Labophot-2A/2 microscope fitted with a 100 W mercury lamp, Nikon UV-1A excitation and barrier filters and an x100 oil objective. Those cells falling within the area of the micrometric ocular in randomly located fields were counted.

3. Results and discussion

Two assays were performed to select the best conditions of pre-treatment. Microorganisms concentrations obtained by direct count in the first experiment and results of One-Sample Kolmogorov-Smirnov Test are shown in table 1. The visualization of microorganisms was improved to shaking and addition of Tween 80 pre-treatment. Since the microorganisms are more separated, and therefore this make easy their counting.

Table 1 Cellular concentration and results of One-Sample Kolmogorov-Sminorv Test.

Pre-treatment	Average	Cell/gVST ($x10^{-9}$)	Kolmogorov-Smirnov Z	Asymp. Sig. (2-tailed)
Without pre-treatment	53.50 ± 11.51	6.57	0.680	0.744
Shaking	63.15 ± 10.28	7.76	0.602	0.861
Tween 80	67.75 ± 12.56	8.32	0.590	0.877
Sonication	48.20 ± 6.13	5.92	0.627	0.826

All the results shown present a normal distribution according to One-Sample Kolmogorov-Smirnov Test. Then, an analysis of variance (ANOVA) was carried out between distinct pre-treatment. It can be concluded that exist significant statistic difference since the significance value for distinct pre-treatment is less than 0.05 (F=14.624 and Sig.=0.00). The results obtained by Paired-Samples T Test are shown in table 2.

There are not significant statistic differences neither between sonication and without pre-treatment nor between shaking and addition of Tween 80. The rest of paired-samples presented significant statistic differences. The interval plots of these paired-samples were shown to select the most appropriate pre-treatment conditions (see figures 1 and 2).

Table 2 Paired-Samples T Test of distinct pre-treatment.

Paired-Samples	95% Confidence Interval of Difference		t	df	Sig. (2-tailed)
	Lower	Upper			
Without pre-treatment-Shaking	-17.79	-1.51	-2.480	19	0.023
Without pre-treatment-Tween 80	-24.02	-4.48	-3.054	19	0.007
Without pre-treatment-Sonication	-0.49	11.09	1.916	19	0.071
Shaking-Tween 80	-12.46	3.26	-1.224	19	0.236
Shaking-Sonication	8.90	21.00	5.170	19	0.000
Tween 80-Sonication	13.43	25.67	6.688	19	0.000

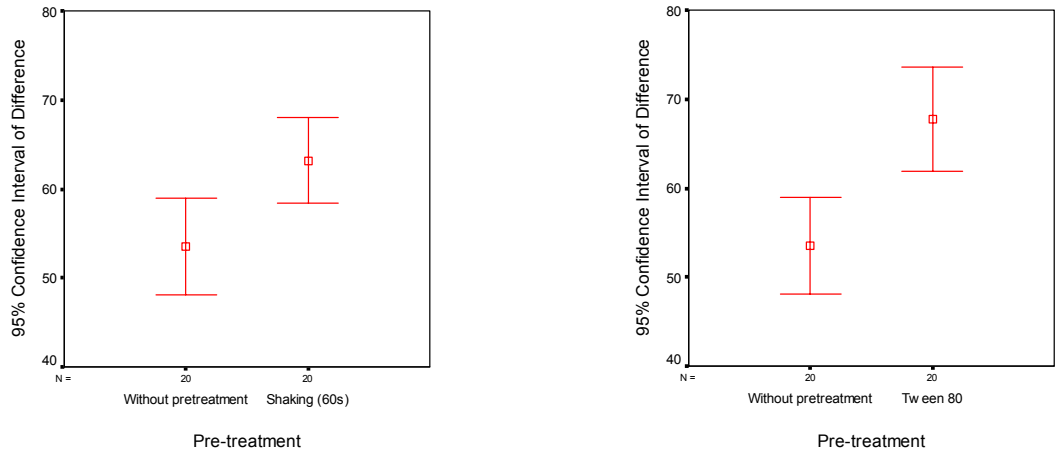

Fig. 1 Interval Plots: shaking and without pre-treatment; Tween 80 and without pre-treatment.

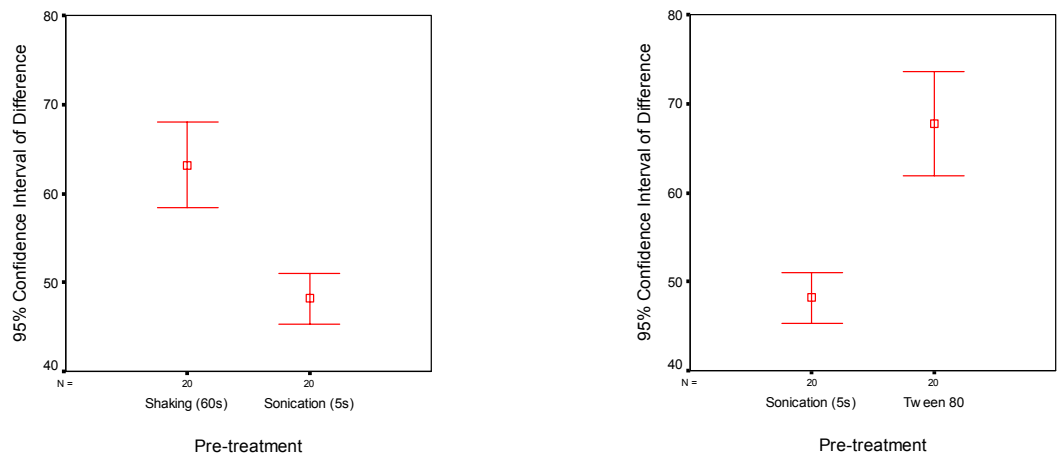

Fig. 2 Interval Plots: shaking and sonication; Tween 80 and sonication.

Finally, it can be concluded that the most appropriate pre-treatment were the addition of Tween 80 together the shaking for 60 seconds.

A new experiment was designed to determine the optimum time of shaking pre-treatment. All the samples assayed contained Tween 80, since this tensioactive agent improves their visualization, increasing their sharpness, and therefore making easy their counting. Microorganisms concentrations, obtained by direct count in the second assay and results of One-Sample Kolmogorov-Smirnov Test are shown in table 3.

All the results shown present a normal distribution according to One-Sample Kolmogorov-Smirnov Test. Then, an analysis of variance (ANOVA) was carried out between distinct shaking times assayed.

It can be concluded that exist significant statistic difference since the significance value for distinct shaking times is less than 0.05 (F=12.814 and Sig.=0.00). An interval plots can be used to select the optimum time of shaking pre-treatment (see figure 3).

Table 3 Cellular concentration and results of One-Sample Kolmogorov-Smirnov Test.

Shaking time	Average	Cell/gVTS ($x10^{-9}$)	Kolmogorov-Smirnov Z	Asymp. Sig. (2-tailed)
20 seconds	30.05 ± 4.69	3.69	0.652	0.789
30 seconds	30.80 ± 4.95	3.78	0.607	0.855
40 seconds	37.20 ± 5.50	4.57	0.833	0.491
50 seconds	31.85 ± 6.66	3.91	0.521	0.949
60 seconds	34.45 ± 4.79	4.23	0.715	0.687
80 seconds	33.15 ± 6.24	4.07	0.788	0.564
100 seconds	42.60 ± 7.42	5.23	0.551	0.922
120 seconds	42.55 ± 6.13	5.22	0.627	0.826
180 seconds	38.80 ± 5.65	4.77	0.732	0.658
240 seconds	37.70 ± 4.17	4.63	0.799	0.546
300 seconds	30.50 ± 7.19	3.75	0.611	0.849

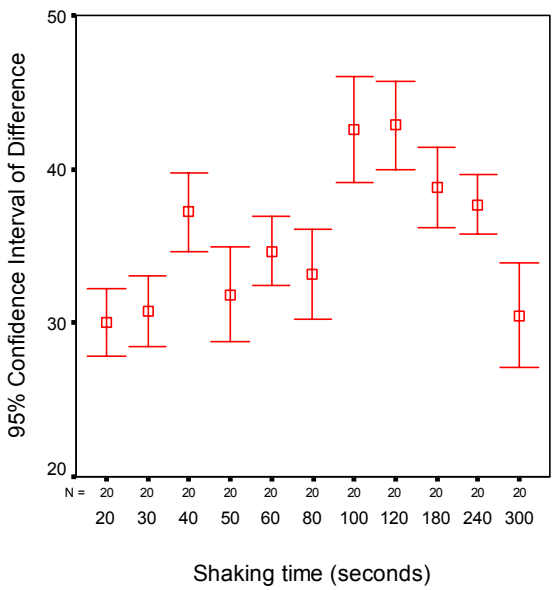

Fig. 3 Interval Plots of distinct shaking times.

4. Conclusions

It can be concluded that the most appropriate pre-treatment applied for microbiological count of high solids content samples was the addition of Tween 80 and 120 seconds of shaking.

The protocol to improve the conditions of environmental samples is of application for fermentations industry, biological treatment of wastes and effluent. Its application is fundamental to control the microorganisms involved in the microbiological process using quantification techniques by epifluorescence microscopy.

Acknowledgements The support by the Spanish Ministry of Science and Technology and Education and Science, projects PPQ2001-4032 and CMT2004-01655 is gratefully acknowledged. B.Montero thanks funding from Council of Education and Science of Junta de Andalucia.

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Environmental, Marine, Aquatic Microbiology.

Geomicrobiology

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A preliminar survey on the fungi of Doña Trinidad Cave, Ardales, Malaga, Spain

F. Stomeo[1,*] G. Ellersdorfer [2] , K. Sterflinger [2], J.M. Gonzalez [1], C. Saiz-Jimenez [1]

[1] Instituto de Recursos Naturales y Agrobiologia, CSIC, Apartado 1052, 41080 Sevilla, Spain
[2] University of Agricultural Sciences, ACBR, Muthgasse 18, A-1190 Vienna, Austria

[*]Corresponding author: e-mail: keka@irnase.csic.es, Phone: +34 954624711

The fungal and yeast diversity of selected soils, animal excrements and air samples from the Cave of Doña Trinidad (Ardales, Málaga, Spain) was investigated by DGGE, RAPD analysis and culturing methods. Fungal and yeast isolates were identified by partial sequencing of their small and large ribosomal RNA subunits (18S and 26S), and internal transcribed spacer regions 1 and 2 (ITS1 and ITS2). Among the isolates members of the genus Fusarium, Arthroderma, Aphanoascus, Aspergillus and Penicillium were detected. In addition to these anamorphs of ascomycetes, representatives of the genus Trichosporon were the only members of the Basidiomycota detected by culturing methods. RAPD patterns of these isolates suggest that they represent different strains within the genus Trichosporon.

Keywords Fungal diversity; Subterranean environment

1. Introduction

Most of the well known rock art caves (Altamira, Lascaux, Tito Bustillo, etc.) are affected by progressive biodeterioration [1-5]. Besides bacteria, fungi have also been shown to play a crucial role in the colonization of caves with prehistoric paintings, such as the case of the Cave of Lascaux, France [6]. Fungi are ubiquitous protagonists in soils and subterranean environments [7] and fulfil a range of important ecological functions although current understanding of fungal biodiversity is limited. Fungi are among the most harmful organisms associated to biodeterioration of organic and inorganic material and their presence in several monuments has been reported [8]. Their metabolic versatility is well known, conferring them the ability to colonize different kind of substrata (wood, glass, rocks, etc.) and to adapt to a wide range of physical and chemical environmental conditions [9]. In this study, we report on the fungal diversity found in a cave containing valuable paleolithic paintings and engravings.

2. Materials and methods

2.1 Studied site

The Cave of Doña Trinidad is located in the town of Ardales, Malaga (Spain) (Figure 1). The cave has a length of 1.5 km with several halls and galleries. The interior contains labyrinths of columns, underground lakes, and beautiful formations of stalactites and stalagmites. Apart from its undoubted interest as a natural phenomenon, the cave's major attractions are the paintings and engravins which date back about 20,000 years from the Upper Paleolithic [10].

2.2 Sample collection

Samples were collected into sterile tubes under aseptic conditions. The fungal diversity of air, three animal excrements, and five soil samples from the Cave of Doña Trinidad (Ardales, Málaga, Spain) were analyzed. Samples were stored on ice and processed as soon as possible, except the samples from the air that were obtained by opening for 10 minutes sterile Petri dishes with a malt extract agar medium, which were directly incubated in the laboratory.

2.3 Isolation and morphological characterization

Fungi were isolated by carefully ground 1g of material which was trasferred to 10 ml 0.01% Tween 80 solution and shaken for 1 h. Different dilutions were prepared and 100 µl of the solution were homogeneously dispersed

onto petri dishes containing culture medium. The media used were 2% malt extract agar (MEA), dichloran rose Bengal agar, Czapek-Dox agar, and yeast extract/peptone/dextrose agar. Incubation was performed at 28ºC for up to one week. Cell counting was carried out. Differential colonies detected on the solid media were transferred to fresh media and isolated by extinction; purity was evaluated by repeated light microscopic observations. Morphological characterization was carried out mounting samples of mycelia over microscope slides and observed microscopically.

2.4 Molecular characterization

Molecular characterization was performed with fungi grown on 2% MEA. Mycelium was scraped from culture plates with a scalpel and trasferred into 1.5 ml Eppendorf tubes containing 500 µl lysing buffer (50mM Tris-HCl, 50mM EDTA, 3% sodium dodecyl sulfate and 1% β-mercaptoethanol, pH 7.5) and 200 µl glass bads (425-600 mm, MERCK, Darmstadt, Germany). The mixture was vortexed in a cell disrupter (Thermo Savant FastPrep, FP120, Holbrook, USA) at full speed for 3 min. After incubation at 65°C for 1h the mixture was vortexed again at full speed for 3 min and then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to a new Eppendorf tube. DNA from the natural soil samples was extracted using the Ultra clean^{tm} soil isolation kit (MOBIO, Carlsbad, CA). DNA quality was determined on 1% agarose gels with TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.0) and visualized after ethidium bromide staining (0.5 mg/ml). Extracted DNA was prepared as a 1:150 dilution to be used in the PCR reactions. PCR was performed using the Master Taq kit. The primer pair ITS1-F (5´ -CTT GGT CAT TTA GAG GAA GTA A -3´) and ITS4 (5´- TCC TCC GCT TAT TGA TAT GC -3´) was used to amplify the ITS regions. For sequencing the 26S rDNA genes the primers used were NL4 (5´ GGT CCG TGT TTCA AGA CGG – 3´) and 18-1184 (5´- GAC TCA ACA CGG GGA ACT C – 3´). DNA was amplified with 35 cycles in a MJ Research PTC 200 thermocycler, as follows: denaturation (94 ºC, 1 min.), annealing (50 °C, 1 min.) and extension (72 ºC, 1 min.), for a total of 35 cycles, followed by a final extension (72 ºC, 5 min.). Amplicons were electrophoresed in 1% agarose gels (Cambrex Bio Science SeaKem LE Agarose) containing ethidium bromide in TAE buffer and photographed. DNA bands were cut off the gels and the PCR products were purified using the Wizard Sv Gel and PCR Clean-up system. Sequencing was performed in an ABI Prism automatic sequencer (Applied Biosystems, CA, USA) following the manufacturer's recommendations. Sequence assembly was done using the Seqman program (Dnastar Inc., Madison, USA). Sequences were submitted for homology searches on line at the National Centre for Biotechnology Infomation, Bethesda, MD. (http://www.ncbi.nlm.nih.gov/BLAST/).

Yeast isolates were differenciated by Denaturing Gradient Gel Electrophoresis (DGGE) as previously described [11, 12] using a GC-rich tailed primer ITS4-GC (5´- TCC TCC GCT TAT TGA TAT GC – 3´ with a GC-rich tail attached at its 5' end) and the reverse primer ITS3 (5´- GCA TCG ATG AAG AAC GCA GC – 3´). Yeast isolates were also analyzed by Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) analysis. Primer PELF (5´- ATA TCA TCG AAG CCG –C- 3´) was used. PCR was performed in a MJ Research thermal cycler (PTC200) programmed for the initial denaturation at 94ºC for 2 min., and 40 cycles of: 94ºC for 34 sec., 45ºC for 70 sec.; 72ºC for 90 sec., with a final extension of 72ºC for 5 min. The isolated strains were conserved in the ACBR (Austrian Center of Biological Resources and Applied Mycology, http://www.biotec.boku.ac.at/acbr.html) culture collection for further investigation.

3. Results and discussion

Sequencing results show a higher variety of fungi in soil and air samples than in excrements. Members of the genera Arthroderma, Aphanoascus, Eurotium, Massarina, Pseudallescheria, Gliomastix, Fusarium, Cladosporium, Penicillium and Aspergillus were found. In excrements the genera Penicillium and Aspergillus were the most abundantly detected among the Ascomycota. Fusarium was detected both in soil and air samples suggesting that it could spread through the cave by air currents formed when opening the cave door. In the Lascaux Cave it has reported a massive invasion of Fusarium, so this fungus might also represent a serious risk for the conservation of Doña Trinidad Cave, if the cave microclimate and environmental conditions change and favour its development. In order to compare molecular and morphological characterization of fungal isolates, some strains were identified morphologically. These strains belonged to the genera Arthroderma, Aphanosascus, Aspergillus and Fusarium according to morphological features. The same isolates identified by molecular methods (i.e., sequencing of their ITS regions) resulted in the detection of homologues to Arthroderma fulvum, Aphanoascus fulvescens, Aspergillus fumigatus, Aspergillus versicolor, Aspergillus sidowii and Fusarium solani. These results lead to a good agreement among these two techniques, suggesting that both methods are useful for the detection of individual isolates and they can be complemented to confirm strain characterization.
In addition to these anamorphs of Ascomycetes, isolates belonging to the genus Trichosporon were detected during this study. This genus is an example of closely related species which delimitation is difficult by currently

accepted methodology [13]. Currently, 21 species are distinguished within this genus each enclosing high diversity and occupying narrowly circumscribed ecological niches [14]. Some species are described to be psychrophilic and inhabitants of soil; some are known to be associated to animals, and five species are of clinical significance [15]. T. laibachii is the most common species of the genus and has a worldwide distribution in soil, sand, mud and plant detritus [16]. It is not human associated and can be clearly distinguished from the pathogenic species T. asahii, T. asteroids, T. cutaneum, T inkin and T. mucoides based on molecular diagnosis.

The DGGE pattern of these isolates using ITS gene fragments allowed to differentiate between the two different genera (Figure 1A). Trichosporon strains have been isolated mostly from two of the three excrement samples. In order to analyze and differenciate them, 26S rRNA gene based and RAPD analysis were carried out. Sequencing results suggest that these isolates could be separated in four major groups related to Trichosporon laibachii, Trichosporon multisporum, Trichosporon akiyoshidainum and Trichosporon sp. Looking at the RAPD patterns performed with primers PELF (Figure 1B) and based on total DNA the Trichosporon isolates clearly represent different strains within the genus. According to RAPD patterns the Trichosporon strains corresponding to lines 1, 4, 5 and 11, and lines 2, 6, 7, 9 and 12, belonged to species of the same genus related to the Trichosporon sp. and Trichosporon laibachii, respectively. Percentages of similarity obtained when comparing 26S rRNA gene sequences of our strains with these two groups of Trichosporon species ranged between 97% and 100%.

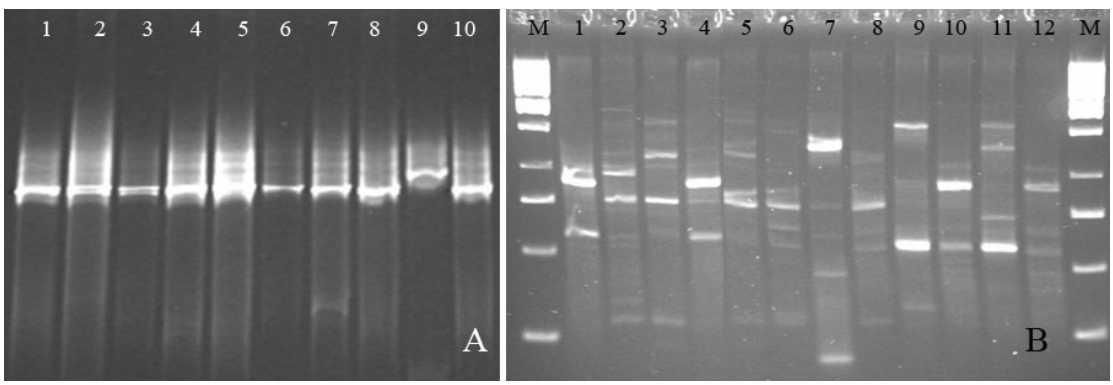

Fig. 1: (A) DGGE pattern of the isolates using ITS gene fragments. 1: AR M13-3.1; 2: AR K11-7; 3: AR M13-5; 4: AR M13-9; 5: AR M13-7; 6: AR M13-8; 7: AR M13-10; 8: AR M13-14; 9: AR M13-4; 10: AR K11-10. Strain AR M13-4 loaded in line 9 is highly related to the genus *Galactomyces geotrichum*. The other strains loaded in the gel are related to different species of the genus *Trichosporon.* (**B)** RAPD pattern with primer PELF of the yeasts strains isolated during this study. First and last lane contains a size marker, 1 kb DNA ladder. 1: AR M13-3.1; 2: AR M13-8; 3: AR M13-9; 4: AR M13-5; 5: AR M13-7; 6: AR M13-10; 7: AR M13-14; 8: AR M13-4; 9: AR K11-10; 10: AR K11-7; 11: AR K11-9; 12: AR K11-11.

Molecular characterization based on rDNA sequences has limited resolution between closely related species suggesting that either morphological determination or the proposal of novel molecular strategies would be of interest to complement and confirm strain differentiation and classification. Thus, at present, the taxonomic rank or species cannot be defined exclusively by using information from the 26S rDNA gene. In this case, RAPD-PCR technique appears to be more sensitive than the rRNA gene sequences for the differentiation of closely related species of this genus. These results suggest the presence of a large diversity within the genus *Trichosporon* in Doña Trinidad Cave.

This study highlights the presence of a large variety of different fungi in this cave which goes well beyond the previously described species suggesting a potential risk as cave colonizers and a potentially negative influence in the conservation of the cave paintings and engravings.

Acknowledgements The authors acknowledge funding from the Junta de Andalucía, Consejería de Cultura through an agreement to study the Cave of Doña Trinidad and to project CGL2006-07424/BOS. FS acknowledge support from the European Community project MEST-CT-2004-513915.

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A survey of microorganisms related to the biodeterioration of prehistoric paintings in natural shelters from Aragon (Spain)

J. M. Gonzalez[1], **R. Alloza**[2] and **M.C. Portillo**[1]

[1] INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGIA, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

[2] LABORATORIO DE ANALISIS E INVESTIGACION DE BIENES CULTURALES, Avda. S. Jose nº 5, nave 6, 50410 Cuarte de Huerva, Zaragoza, Spain

The region of Aragon, on Northeastern Spain, presents an unusual richness of prehistoric paintings spread all over its territory. These paintings correspond to a wide chronological period ranging from the upper paleolithic to the recent prehistoric times. Current initiatives to preserve these shelters for future generations, are focusing on understanding the biogeochemical processes going on at these sites as a necessary step to better preserve these paintings. This study focuses on the microbiology of these shelters. Molecular methods based on the use of nucleic acids were used to determine the microorganisms inhabitants of these sites. Molecular techniques are culture-independent methods. In this study, molecular techniques based on both DNA and RNA were utilized, so the cells present in these environments as well as the metabolically active microorganisms, respectively, can be differentiated during the experimental procedure.

Keywords biodeterioration; microbial communities; prehistoric paintings

1. Introduction

Microorganisms cause a variety of biodeteriorating effects on cultural heritage. The importance of microorganisms in deterioration has been demonstrated in the last years [1-3]. The development of complex microbial communities in caves, mural paintings from catacombs and other monuments have been reported [4-9]. However, the microbiota from natural shelters with prehistoric paintings has not been approached and might present specific characteristics.

Culture-independent methods, such as molecular techniques based on nucleic acid analysis, allow the detection of microorganisms within complex microbial communities without the need for previously growing the microorganisms to be detected in the laboratory. Using DNA and RNA the microorganisms present (based on DNA) in a microbial community and the fraction of that community showing significant metabolical activity (based on RNA analysis) [7, 10, 11] can be surveyed. Despite the reported complexity of natural microbial communities, including those developing on caves with prehistoric paintings [4-8, 11], molecular methods permit to identify the major components of these communities even if a large majority of the microorganisms in natural systems can not generally be cultured.

This study focuses on identifying the major components of the microorganisms associated to salt precipitates developing at the walls of a natural shelter with numerous representations of prehistoric art. Molecular methods based on DNA and RNA were used to identify the microorganisms involved a biodeteriorating process in order to approach adequate conservation strategies.

2. Materials and Methods

Aragon, located on Northeastern Spain, presents a large number of sited with prehistoric paintings mostly located in natural shelters throughout its territory. In this study, we have studied the microorganisms involved in a biodeteriorating phenomenon, the precipitation of salts and formation of whitish areas on the walls of a natural shelter containing numerous paintings and engravings. This shelter is named Fuente del Trucho and it is located in the municipality of Colungo (Huesca, Aragon) (Figure 1).

Visual inspection of the shelter lead to the identification of whitish spots showing precipitation of salts and the exfoliation of the superficial rock layer (Figure 2). Samples were collected and immediately preserved in RNAlater solution (Ambion, Austin, USA), mantained refrigerated until arrival to the laboratory where they were stored at -80ºC.

The major components of the microbial communities were analyzed by molecular methods based on DNA and RNA. DNA will provide with information n the microorganisms present in the analyzed samples while using RNA those microorganisms showing significant metabolical activity within the community will be detected. The methods used have been previously described [7, 11, 12] and DNA and RNA extraction, PCR

amplification of 16S rRNA gene fragments, cloning, clone screening, sequencing, and sequence data analysis have been performed as reported therein. Microbial communities fingerprinting was performed as described by Gonzalez and Saiz-Jimenez [3] by Denaturing Gradient Gel electrophoresis (DGGE) and were used to characterized the major microbial components of the studied communities. Microorganisms identified through 16S rRNA gene fragment sequencing were located at the migration level in DGGE profiles.

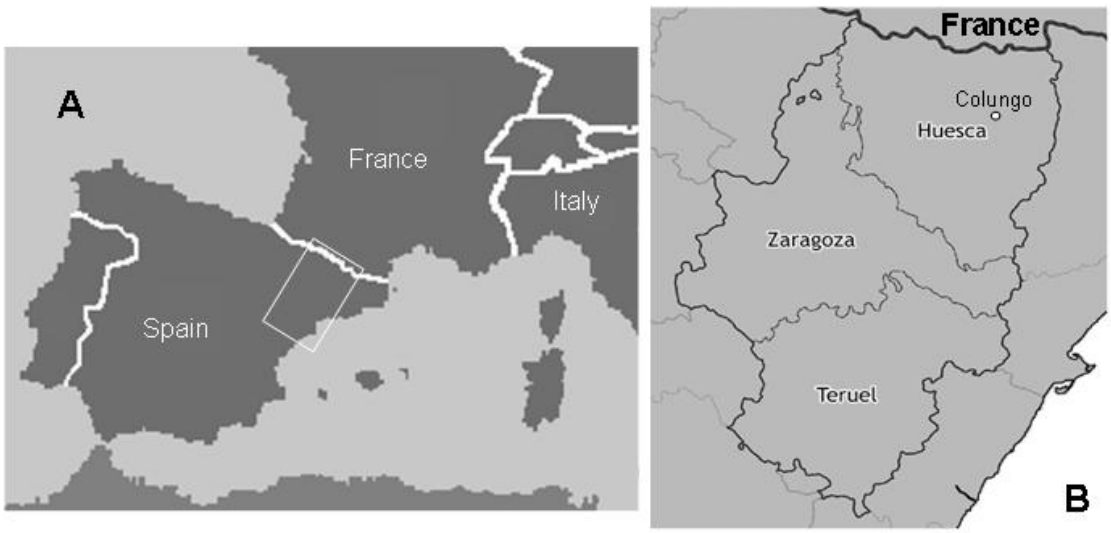

Fig. 1 Location of the natural shelter Fuente del Trucho in Colungo (Huesca, Aragón, Spain). This natural shelter contains numerous prehistoric paintings and engravings dated from the Neolithic period. An example of these representations is shown in panel B.

3. Results and Discussion

Visual inspection of whitish precipitates due to salt deposition revealed that these areas were related to the exfoliation of the upper rock layer of some areas of the walls of the studied shelter. This study focuses on the analysis of the bacterial community associated to these deposits as a first step in understanding the development of this precipitation process. The bacterial group most frequently detected based on DNA analysis were the Gammaproteobacteria, followed by Betaproteobacteria and Alphaproteobacteria. These bacterial phylla represented the bacteria more characteristics among those present at the studied spots. In order to determine if these bacteria showed significant metabolical activity within its community, we analyzed these samples based on RNA extraction, reverse transcryption, PCR amplification, and DGGE fingerprinting and clone sequencing. Results showed that the metabolically active fraction of the community were mostly represented by Gammaproteobacteria (67.4% of total RNA sequences), followed by Betaproteobacteria (18.6%) and Alphaproteobacteria (7.0%), Actinobacteria (4.6%), and Bacteroidetes (2.3%). Thus there was an agreement between the major bacterial groups showing presence and metabolic activity at these sites. Other bacteria were detected as well but represented lower fractions of the community than the previous ones and constituted less than 5% of total. Thus, the Proteobacteria, represented by member s of the Gamma, Beta and Alpha subphylla were the most abundant and metabolically active bacteria in these samples, and consequently, the bacteria directly implicated in inducing the precipitation process as previously suggested [13, 14].

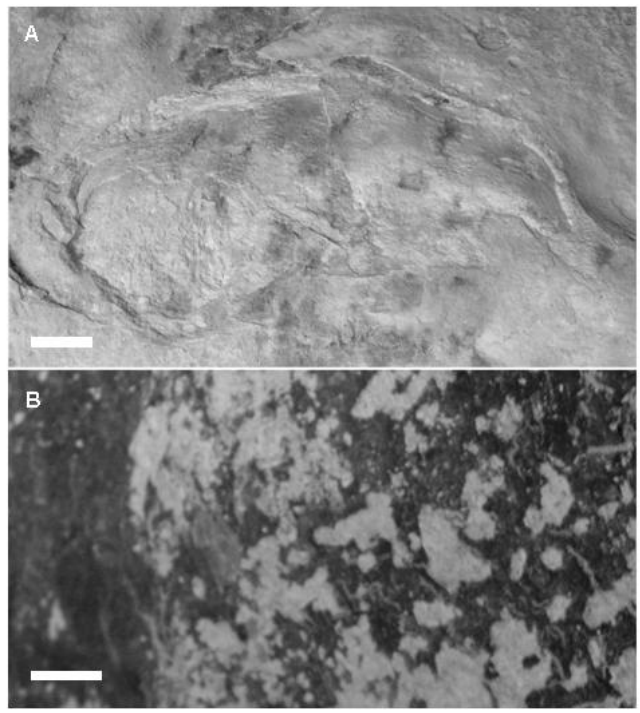

Fig. 2 An example of engraving from the natural shelter Fuente del Trucho representing a resting bear (A). A photograph of the whitish zones studied in this work (B). Bars indicate 10 cm in A and 2 cm in B.

Table 1 Major components of the bacterial community from salt deposits detected during this study. The percentage shown correspond to the fraction of total sequences processed in this work. Results from both DN- and RNA-based molecular analyses are compared.

	Percentage of sequences	
Bacterial group	DNA	RNA
Gammaproteobacteria	57.1	67.4
Betaproteobacteria	28.6	18.6
Alphaproteobacteria	14.3	7.0
Actinobacteria	-	4.6
Bacteroidetes	-	2.3

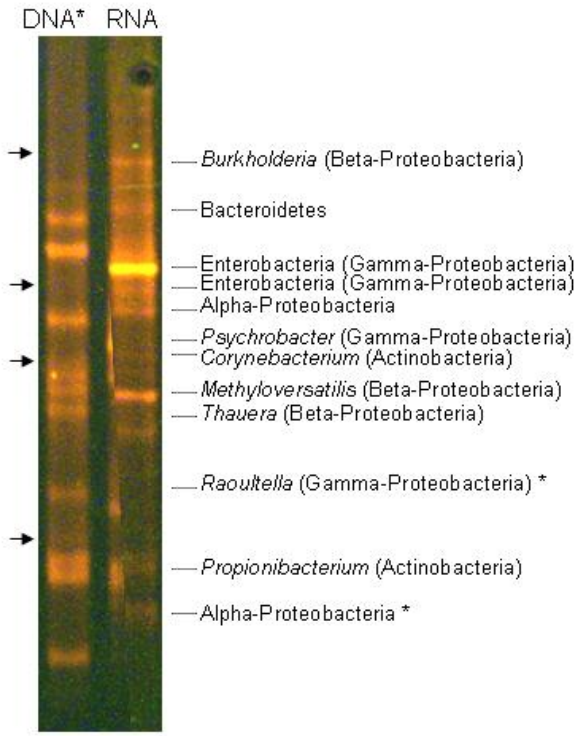

Fig. 3 Microbial community fingerprint obtained by PCR-DGGE based on DNA and RNA. The microorganisms identified in these samples are indicated at their level of migration during the DGGE analysis. Asterisks indicate the identification of microorganisms based on DNA analysis, not labeled identifications were obtained from RNA analysis.

In spite of the agreement mentioned on the results ontained from DNA- and RNA-based molecular analyses, microbial community fingerprints revealed clear differences among the communities of microorganisms present (DNA-based) and those metabolically active (RNA-based) (Fig. 3). Thus, even if the same groups are represented through both (RNA and DNA) analyses, the representatives from each of them were different and so, the bacterial community structure of present microorganisms could be distict of the community of metabolically active bacteria. Bacterial community fingerprints (Fig. 3) showed that the major component of the metabolically active community was a representant of the Gammaproteobacteria, specifically within the Enterobacteria. A second band showing significant signal in the elctrophoresis analysis corresponded to a Betaproteobacteria related to the genus *Methyloversatilis*. Thus, the two major components of these bacterial communities were a heterotrophic, anaerobic facultative bacteria (Enterobacteria) and an aerobe characterized by a methilotrophic metabolism (*Methyloversatilis*).

Other bacteria were detected in this study as members of the studied community. These results were obtained from the sequencing of clones from the 16S rRNA gene libraries constructed during this study. Most Gammaproteobacteria detected in this study belowng to the Enterobacteria. Sequences related to the genera *Psychrobacter* and *Raoultella* were detected. Among the Betaproteobacteria, *Methyloversatilis, Thauera,* and *Burkholderia* were the more representative genera. Interestingly, the genus *Thauera* corresponds generally to anaerobes, suggesting the existance of anaerobic microniches within these sites. The Alphaproteobacteria detected were no closely related to described genera and so their participation on these communities remains unknown. The Actinobacteria were also represented in these samples although constituted less than a 5% of total processed sequences. The genera detected in these samples from the Actinobacteria were *Propionibacterium* and *Corynebacterium*, common components of soil and rock bacterial communities.

A community based on heterotrophic components constituted the analyzed samples. Bacteria detected during this study were associated to the precipitation of salts and likely induced its deposition [13, 14]. This can be achieved by fovouring pH increases as a result of producing a global alkalinization of their microenvironment. Metabolic products or consumption of other microorganisms products will lead to a global pH increase or decrease. In the case of carbonate depositions, pH increase is required for carbonate precipitation.

The bacterial communities detected during this work suggest the participation of different bacteria, being the Proteobacteria the predominant component, in inducing salt precipitation and deposition at the studied shelter. Further investigation on the physiology of these bacteria and their effect on salt precipitation might lead to a better understanding of the deposition process of salts at the studied shelter and deduce possible strategies of conservation of the paintings and engravings contained it the walls of Fuente del Trucho shelter.

Acknowledgements The authors acknowledge support from the Aragon Government under project PM055/2006.

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An Investigation on Heterotrophic and Pathogenic Gastrointestinal Bacteria in Otamiri River, Nigeria

Chinedu William Agbakwuru

Department of Microbiology, Faculty of Sciences, Imo State University, Owerri Imo State, Nigeria

A study was completed to estimate the numbers of heterotrophic and pathogenic gastrointestinal bacteria in the Otamiri River Nigeria. Samples were collected at three stations (A,B,C) in the Owerri Municipal Council area of Imo State. Heterotrophic and coliform bacteria were plated on nutrient and MacConkey agar. Results indicated heterotrophic bacterial counts of $4.7x10^3$, $4.7x10^1$, $3.4x10^1$ cfu/ml for Stations A,B, and C respectively. Coliform counts were $2.7x10^3$, $2.7x10^2$ and $2.3x10^1$ cfu/ml for Stations A,B, and C respectively. Physiochemical identification tests on isolates had values within the standard range and revealed *Citrobacter Sp., Streptococcus Sp., Salmonella Sp., Enterobacter Sp., Lactobacillus Sp., Proteus Sp., Escherichia Sp., Pseudomonas Sp., Shigella Sp., Vibrio Sp.,* and *Bacillus Sp.,*Gastroenteric bacteria isolated were *Salmonella Sp., Shigella Sp., Vibrio Sp.,* and *Escherichia Sp.,* and probably resulted from untreated sewage and/or non-point source pollution entering the river.

Keywords Bacteria outnumber, outweigh, out-travel, and outevolve us

Introduction

Water pollution occurs when a body of water is adversely affected due to the addition of large amounts of materials to the water [26]. When water is unfit for its intended use, water is considered to be polluted [14]. Water pollutants exist in two types Viz: Point source and Non-point source.

Point source occurs when harmful substances are emitted directly into a body of water while a non-point source delivers pollutants indirectly through environmental changes. This study is aimed at analyzing water samples from Otamiri River, to determine the level of chemical and microbiological quality of the water which will help estimate the level of contamination and determine the safety of the water for domestic use, since Otamiri River serves as source of water for communities around.

Materials and method

A source of bacterial sample for this study is river water obtained from Otamiri River located about 50 meters west of Owerri Municipality. Surface water samples were collected with sterile bottles from three different locations. These include Station A (Opposite total filling station, Aba Road), Station B behind timber shade), Station C (Behind Emmanuel College). These samples were transported to the laboratory for analysis. All glass wares were sterilized using dry heat in an electric hot oven at 160^0C for 2 hours [22]. The media and diluents used were sterilized at 121^0C for 15 minutes. Wire loop and inoculating needle were sterilized by flaming in a naked gas flame until red-hot. Glass rods were further sterilized by dipping in alcohol and flaming in a Bunsen flame during streaking. The diluents used are normal saline, which was prepared by dissolving 8.5g NaCl (BDH) in 1 liter of water. Ten- fold serial dilution of the samples was carried out using physiological normal saline as diluents. 0.1 ml of each dilution was inoculated in triplicates on Nutrient Agar, MacConkey, SSA and TCBS Agar and incubated at 37^0C for 24 hours. Number of colonies that appeared after incubation was subculture on a fresh Nutrient Agar medium for purity and then transferred onto slants, stored at 4^0C in a refrigerator for further use. Bacterial isolates were identified using macroscopic and microscopic examination of the cultures followed by biochemical tests. Gram staining method described by Cruickshank *et al;* [13] was adopted. Thin smear of the isolates were made on a clean grease free glass slide. Heat fixed by passing Bunsen burner flame for 2-3 times then flooded with malachite green and steamed for minute by heating over a beaker of boiling water. Rinsed in distilled water and flooded with saframin for 20 seconds and later washed in tap water, blotted dry and then examined under oil immersion objectives lens. Motility test as described by Ogbulie *et al;* [22] was used to differentiate motile and non-motile bacteria. Parameters determined include Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), pH, and Temperature.

Results

Total heterotrophic bacterial and coliform counts result of the water samples are shown in table I. From the results, sampling Station A has the highest bacterial and coliform of $4.7x10^3$ cfu/ml and $2.7x10^3$cfu/ml respectively, followed by samples from Station B, which gave bacterial and coliform counts of $4.7x10^1$ cfu/ml and $2.7x10^2$ cfu/ml respectively. Station C gave counts of $3.4x10^1$ cfu/ml and $2.3x10^1$cfu/ml for bacterial and coliform respectively. A total of 20 bacterial isolates were obtained from water samples collected from the three different sampling stations along Otamiri River. The result indicates that out of the 20 isolates, 12(60%) are gram negative rods which include *Citrobacter Sp., Enterobacter Sp., Klebsiella Sp., Protus Sp., Escherichia Sp., Vibrio Sp., Pseudomonas Sp., SerratiaSp.,* and *Shigella Sp.,* 6(30%) are gram positive rods which include *Salmonella Sp., Lactobacillus Sp.,* and *Bacillus Sp.,* while 2(10%) are gram positive cocci which include *Streptococcus Sp.,* and *Staphylococcus Sp.,*. From the results shown in table II, pathogenic isolates make up 10 (50%) of the 20 isolates obtained which include *Salmonella Sp., Vibrio Sp., Shigella Sp.,* and *Escherichia Sp.,* and probably resulted from untreated sewage and /or non-point source pollution entering the river. Results obtained from the physical and chemical analysis shown in table III shows that BOD, COD, pH, and Temperature are 47.00mg/L,26.00mg/L,6.3 and 22.8^0C respectively.

Table I: Microbial load of the water samples.

S/N	SAMPLING STATIONS	TOTAL HETEROTROPHIC BACTERIAL COUNT (cfu/ml).	TOTAL COLIFORM COUNT(cfu/ml)
1	Station A: (Opposite Total gas station, Aba road).	$4.7x10^3$	$2.7x10^3$
2	Station B: (Behind Timber shade).	$4.7x10^1$	$2.7x10^2$
3	Station C: (Behind Emmanuel College).	$3.4x10^1$	$2.3x10^1$

Table II: Gastrointestinal pathogenic bacteria isolated and percentage occurrence

ISOLATES	% OCCURRENCE
Salmonella Sp.,	4(40%)
Shigella Sp.,	2(20)
Vibrio Sp.,	1(10)
Escherichia Sp.,	3(30)
Total	10

Table III: physical and chemical parameters

PARAMETERS	VALUES
BOD (mg/l)	47.00
COD (mg/l)	26.00
pH	6.3
Temperature (^{0}c)	22.8

Discussion

Highest bacteria count was observed at station A which receives the highest refuse disposal and gets more polluted as most industries discharge their effluent. Followed by Station B which although, is relatively high receives sawdust and other wood particles as its effluent from timber shade. Samples taken from station C show low bacterial presence. This would be as a result of the absence of human activities in these sites and the inevitable influence of human activities in these sites and the inevitable influence of water dilution as reported by other workers [6]. The high bacterial counts obtained in this study could be influenced by the existing environmental conditions. This is in view of the fact that these samples were collected during dry season months of November through January which have been reported by Barrd and Powland [4] to influence the isolation of

bacterial groups in the tropics. This is in view of the fact that Nigeria like other countries in the tropics with pronounce dry season may have concentrated water and other factors. Similar concentrations of water have been reported by Okpokwasili and Ogbulie [23] to account for increased bacteriological and physio-chemical quality of river. The fundamental facts of this study is that the river seems to be biologically polluted during dry season months of November through January which may unravel the high risk of water borne diseases during this period. Regrettably, the period of November and January, during which the organism were isolated coincides with the period of least water availability for personal hygiene.

Conclusion

Clearly, the problems associated with river pollution are the capabilities to disrupt life on our planet to a greater extent. Congress has passed laws in order to combat river pollution thus acknowledging the fact that river pollution is indeed a serious issue. But the government alone cannot solve the entire problem. It is ultimately up to us to be informed, responsible and involved when it comes to the problems we face with our river. We must become familiar with our local river "Otamiri", its resources and learn ways of disposing harmful household wastes so that they don't end up in sewage treatment plants that can't handle them or landfills not designed to receive hazardous materials. As we head into the 21st century, awareness and education will most assuredly continue to be the two most pollution. If these measures are not taken and river pollution continues, life on earth will suffer severely. Global environmental collapse is not inevitable. The developed World must work with the developing World to ensure that new industrialized economies do not add to the world's environmental problems. Politicians must think of sustainable development rather than economic development. Conservation strategies have to become more widely accepted and people must learn that energy use can be dramatically diminished without sacrificing comfort. In short, with the technology that currently exists, the years of global environmental mistreatment will be a thing of the past.

Acknowledgement Thanks goes to my parents,Cecilia Adanma Eberendu-ogu and family for their assistance ,my fiancé Chidinma L.S. Iwu,Prof.G.Lanza,Prof.B.E.B.Nwoke of Imo state university and all my friends for their encouragement.

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Assessment of the performance of porphyrin derivatives as photosensitizers for the inactivation of bacterial endospores

A. Oliveira[1], **A. Almeida**[1], **C. Carvalho**[2], **J. Tomé**[2], **M. A. F. Faustino**[2], **M. G. Neves**[2], **A. C. Tomé**[2], **J. Cavaleiro**[2] and **A. Cunha**[*,1]

[1] Department of Biology & CESAM, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal.
[2] Department of Chemistry, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal.

[*]Corresponding author: e-mail: acunha@ua.pt, Phone: +351 234 370 784

Spore-producing bacteria have proved to be extremely resistant to solar and photocatalytic disinfection techniques. Porphyrin derivatives that produce active oxygen species in the presence of light and molecular oxygen can be an interesting approach for the inactivation of these highly resistant bacterial spores. This work reports on studies of photodynamic inactivation (PDI) of *Bacillus cereus* endospores, taken as model-endospores, using several porphyrin derivatives, differing in the number of positive charges. The results show definitively and contrary to what was previously reported in the literature, that porphyrin derivatives are effective photosensitizers for the inactivation of bacterial endospores making PDI a promising approach for the disinfection of living tissues, contaminated materials and wastewater.

Keywords photodynamic inactivation; bacterial endospores; porphyrin derivatives

1. Introduction

The production of endospores allows bacteria to survive in extreme environmental conditions for long periods of time, having a major ecological importance [1]. Bacterial endospores have a complex multilayered coating that is highly resistant to inactivation by alcohols, phenols, chlorhexidine and benzalkonium compounds, which easily destroy vegetative cells of bacteria [1]. Photodynamic inactivation (PDI) uses non toxic photosensitive substances, namely phenothiazines and porphyrins [2]. When irradiated with visible or ultraviolet light, these substances absorb radiation of adequate wavelength, suffering an electronic excitement and reacting with oxygen molecules that rapidly react with its environment (cell wall material, nucleic acids, peptides, lipids) leading to cytotoxicity [2]. The use of photosensitizers (PS) is now regarded as an innovative approach in the search for new efficient and ecologically sustainable antimicrobial agents, applied to the environment, human health and food safety.

In this work we propose to evaluate the applicability of cationic porphyrin derivatives, differing in the number of positive charges, in the inactivation of bacterial endospores.

2. Materials and Methods

Bacillus cereus (ATCC14579) pure cultures and endospores stock suspensions were prepared according the procedure used by Demidova and Hamblin [1].

The photosensitizers tested in this study are presented in Figure 1: (i) 5-(1-methylpiridinium-4-yl)-10,15,20-tris(4-carboxyphenyl)porphyrin iodide (Mono-Py+-COOH, monocationic), (ii) 5,10-bis(4-carboxyphenyl)-15,20-bis(1-methylpiridinium-4-yl)porphyrin di-iodide (Di-Py+-Me-COOH cis, dicationic), (iii) 5-(4-carboxyphenyl)-10,15,20-tris(1-methylpiridinium-4-yl)porphyrin tri-iodide (Tri-Py+-Me-COOH, tricationic); (iv) 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py+-Me, tetracationic) and (v) toluidine blue O (TBO). TBO was tested as a reference PS, since its effectiveness against *B. cereus* endospores is documented in the literature [1].

As light source, an optical fibre illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp (400-800 nm) was used. Irradiation (1690 w m^{-2}) was conducted continuously during 4-10 minutes.

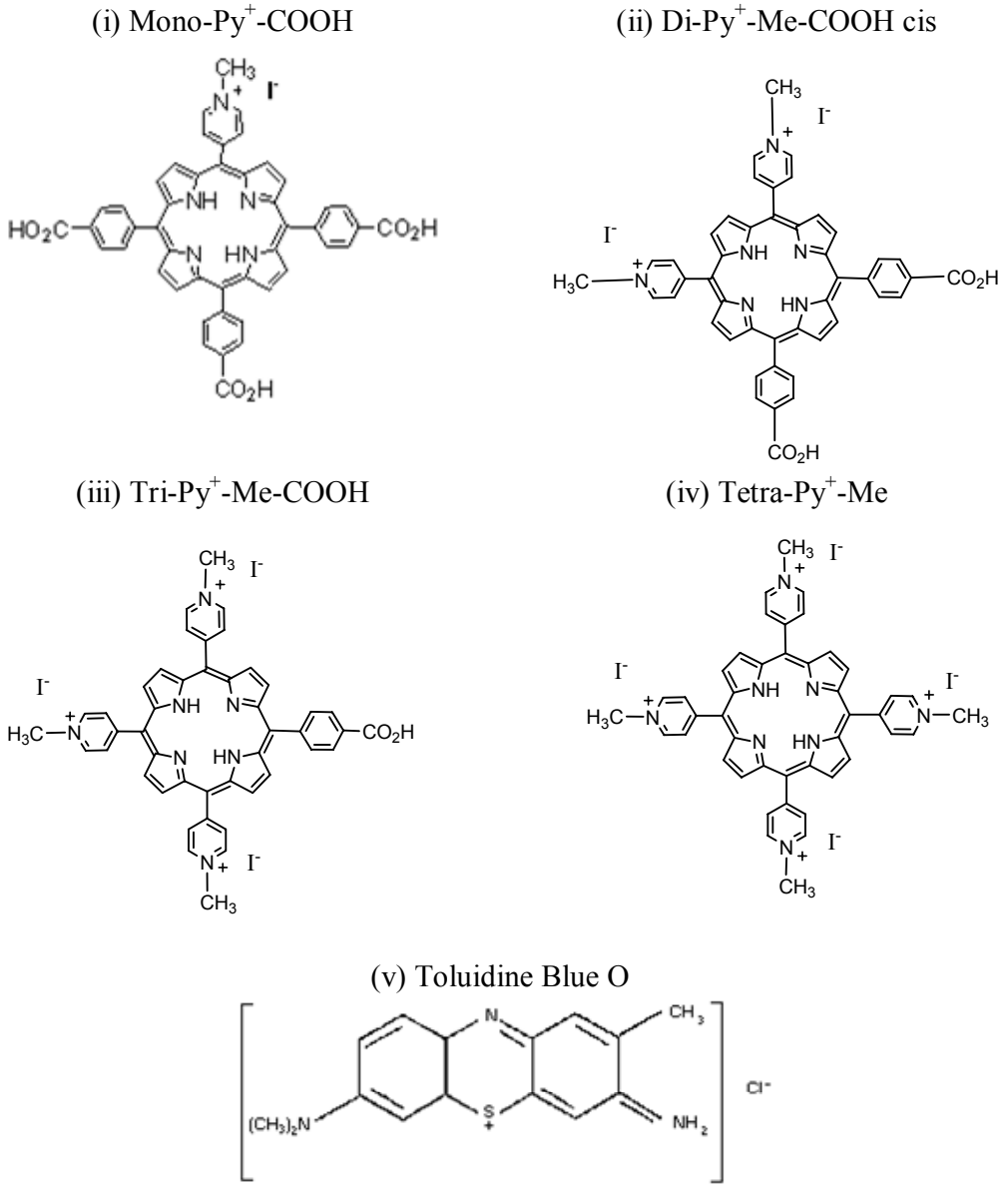

Fig. 1 Structures of the tested photosensitizers.

Treatments were initiated with a pre-exposure step, with the aim of maximizing the adsorption of PS material to the spore surface and simultaneously reduce colour intensity during the irradiation and minimize self-shading. Aliquots of 1 mL of spore stock-suspension were transferred to a series of sterile microtubes and incubated in the dark, at 37 ºC, for 3 hours in the presence of 10, 30 and 60 µM of PS. After incubation, suspensions were centrifuged 10 minutes at 1,550 x g (Eppendorf Microcentrifuge 5414). The pellet was resuspended in 1 mL of PBS and homogenised in the vortex. For the irradiation, the content of each microtube, corresponding to the different treatments/concentrations, was added to 9 mL of PBS and irradiated under magnetic stirring. During the irradiation, samples were kept in ice to prevent heating. Two controls were included in each irradiation experiment: one light control (LC) submitted to the same irradiation conditions as the samples but in the absence of PS and one dark control (DC) containing the highest concentration of PS tested in each experiment, but protected from the light with aluminium foil. The inactivation was followed by collecting, serial-diluting and pour-plating in TSA (Tryptic Soy Agar, Difco Laboratories) triplicate 100 µL aliquots at the beginning of the experiment and at selected time intervals during the irradiation. Colonies were counted after 48 hours of incubation at 30 ºC, in the dark and the inactivation was estimated from the relative variation of the concentration of colony forming units (CFU).

3. Results

The results of the PDI experiments with *B. cereus* are presented in Figure 2. The maximum inactivation was achieved with TBO. A maximum reduction of 3.9 log units relatively to the initial concentration of endospores was observed with a concentration of 30 μM and an irradiation period of 10 minutes. With 60 μM of TBO, a reduction >3 log units was observed immediately after the first minute of irradiation.

Monocationic and dicationic porphyrins induced minor endospore inactivation, respectively 0.4 and 0.5 log units. Tricationic porphyrin caused marked reductions of the concentration of viable endospores. A 3.1 log unit reduction was obtained with the tricationic compound. With the highest PS concentration (60 μM) a reduction of 3 log units was obtained after 1 minute of irradiation. With Tri-Py^+-Me-COOH, the 10 μM concentration caused an inactivation that was equivalent to that of the 60 μM concentration, after 5 minutes of irradiation. The tetracationic porphyrin Tetra-Py^+-Me caused a maximum reduction of 2.4 log units with a 60μM concentration, after 2 minutes of irradiation.

The profiles of inactivation of all tested PS show that the effects occurred mostly during the first 2 minutes of irradiation.

4. Discussion

The inactivation of bacterial endospores has been regarded as a challenge for human health, environmental quality and food safety. PDI approaches had been already attempted but with limited success as to the use of porphyrin derivatives as photosensitizers. However, the effects of modifications in the molecule charge and in the nature of the meso-substituent groups on it effectiveness and selectivity towards different bacterial groups are well documented [3]. For this study we started with the hypothesis that modifications of the porphyrin derivatives could improve their photo induced inhibitory potential towards bacterial endospores. Several porphyrin derivatives differing in the number of charges were tested and the PDI effects were interpreted in comparison with those obtained with TBO, an effective PS for *B. cereus* endospores [1]. According the maximum reduction in the concentration of viable endospores, the tested PS could be organized by the following order of decreasing effectiveness: TBO>Tri-Py^+-Me-COOH>Tetra-Py^+-Me>>Mono-Py^+-COOH=Di-Py^+-Me-COOH.

Cationic porphyrins have been found to photoinactivate gram positive bacteria and also gram negative strains, better than the neutral molecules [4-6], because of improvement of the interactions with the rather impermeable outer membrane [4-5]. However the relation with the molecule's charge is not linear. In this study, the tricationic derivative was more effective than the tetracationic one. Demidova and Hamblin [1] had tested without success, a tricationic porphyrin [5-Phenyl-10,15,20-tris(N-methyl-4-pyridil)porphyrin chloride] in their pioneer work of PDI directed to bacterial endospores. The tricationic compound used in our study differs considerably from the former product confirming that changes in the molecule significantly affect interactions between the PS and the target structures or compounds [4].

5. Conclusion

From this work we can anticipate that suitable modifications of the porphyrin charge and structure may significant improve its PDI potential for endospores inactivation. Since the tricationic derivative was the most effective against *B. cereus* endospores and showed no signs of toxicity in the absence of light, it should be regarded as worthy to explore for PDI of spore-producing gram-positive bacteria.

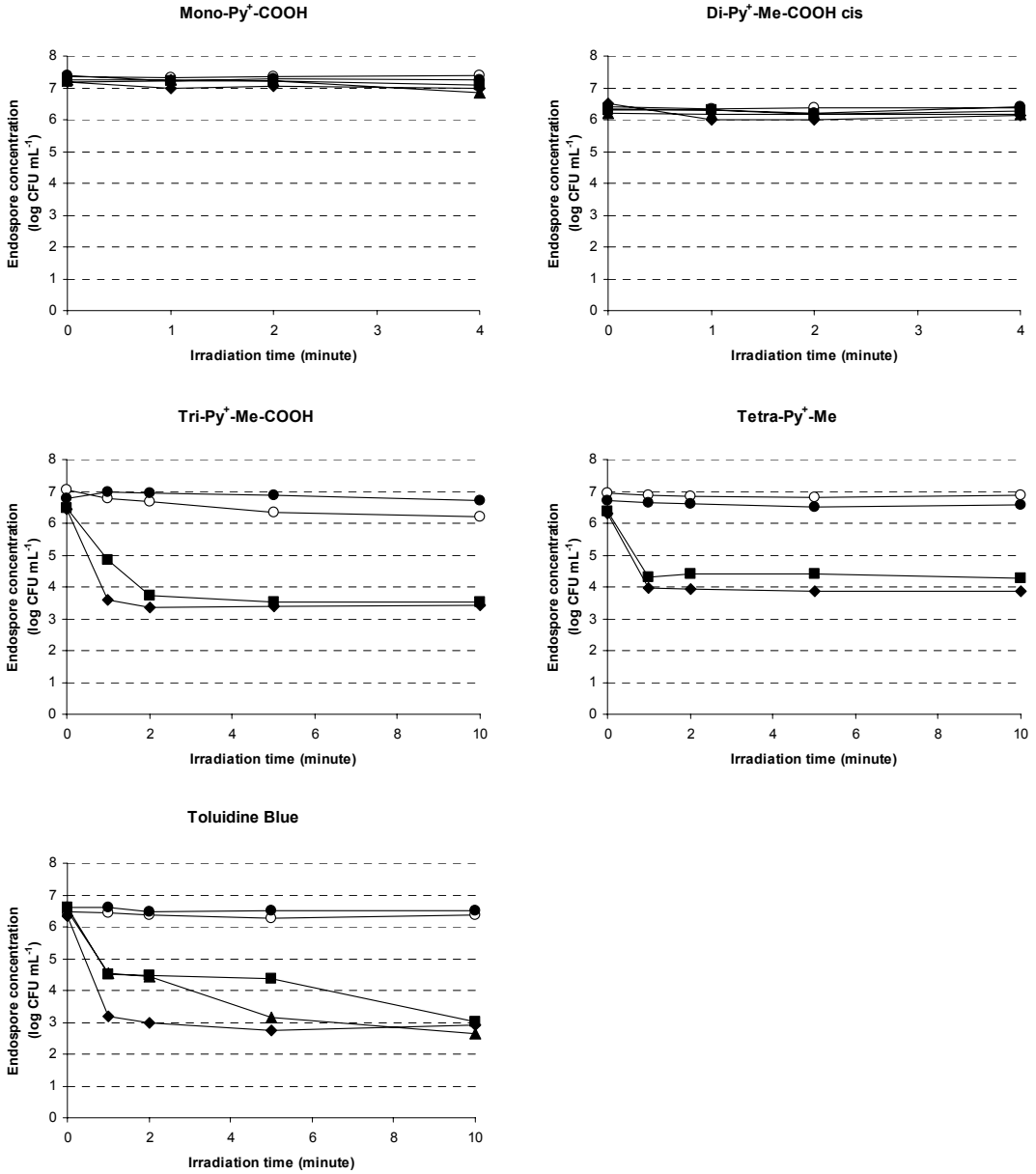

Fig. 2 Survival of *B. cereus* endospores during PDI experiments with 10, 30 and 60 μM of PS. —○— LC, —●— DC, —■— 10 μM, —▲— 30 μM, —♦— 60 μM.

Acknowledgements Thanks are due to the University of Aveiro, FCT and FEDER for funding the Organic Chemistry Research Unit, and to the project POCI/CTM/58183/2004 and CESAM for funding the Microbiology Research Group. C. Carvalho and J. Tomé are also grateful to FCT for their grants (SFRH/BPD/5630/2001).

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Biodegradation of aromatic contaminants present in industrial production waste waters by *Trichosporon cutaneum*

Z. Alexieva[*,1], M. Gerginova[1], P. Zlateva[2], D. Ivanova[3], N. Peneva[1], B. Atanasov[1]

[1] Institute of Microbiology, Bulgarian Academy of Sciences, Acad.G.Bontchev str., bl.26, 1113 Sofia, Bulgaria
[2] Institute of Control and System Research, Bulgarian Academy of Sciences, Acad.G.Bontchev str., bl.2, 1113 Sofia, Bulgaria
[3] University "Prof. Asen Zlatarov", Prof. Yakimov str., 1, 8010 Bourgas, Bulgaria

[*]Corresponding author. Tel.: +35929793120; Fax: +35928700109; *e-mail*: zlatkama@microbio.bas.bg

The microbial strains used for decontamination of different origin wastewater should not only be highly active to one of the contaminants but they should also be resistant enough to the remainder. Their resistance can be ensured by the degradation activity of the strains used towards most of the waste products present in the wastewater. *Trichosporon cutaneum* R57 is known as an effective biodegradant able to utilize and thus remove a number of toxic aromatic compounds from the environment. The present paper deals with processes of degradation and utilization of monohydroxyl derivatives of phenol (resorcinol, catechol and hydroquinone), as well as some of the most toxic aromatic pollutants of the environment like 2,6 - dinitrophenol, α-methylstyrene and acetophenone. The basic kinetic parameters for the biodegradation of the listed above compounds are reported. The highest initial concentrations which could be degraded by the investigated strain were as follow: resorcinol – 1.6 g/l; catechol – 1.3 g/l; hydroquinone – 1.2 g/l; 2,6-dinitrophenol – 0.7 g/l; α-methylstyrene and acetophenone – 0.5 g/l. The inhibition coefficients were calculated according to the Haldane-kinetics. The results obtained certainly proved the ability of strain *T. cutaneum* R57 to degrade wide range toxic contaminants present in industrial production wastewaters.

Keywords *Trichosporon cutaneum*; biodegradation; aromatic compounds; growth kinetics

1. Introduction

The wastewaters from industrial facilities for production of phenol contain a number of toxic organic compounds. Beside phenol itself, they usually contain acetophenone, α-methylstyrene, benzoic acid, dimethyl phenyl carbinol, methanol and isopropylbenzene. The removal of most of these compounds usually requires complex physico-chemical treatment of the wastewaters and is not quite effective. The following biological treatment where the main components are microorganisms becomes still more important [1, 2]. The metabolism of aromatic compounds, particularly phenol and its derivatives, has been intensely studied with prokaryotic microorganisms [3-6].

Several yeast species of genera: *Candida, Rhodotorula* and *Trichosporon* which can biodegrade aromatic compounds have been described in the literature [7-10]. Yeast strains belonging to genus *Trichosporon* have been isolated from various sources: soil, industrial wastewaters, ligneous pulp, activated sludge and were reported to degrade a number of aromatic compounds [11-14].

The object of our investigation, strain *Trichosporon cutaneum* R57 is distinguished among most of the strains discussed in the literature for its ability to degrade high concentrations of phenol (up to 1g/l) as well as many other highly toxic aromatics for a quite short time (16-27h) [15-17]. The possibility for covalent immobilization of strain cells makes it a perspective object for development of continuous technologies for purification of wastewaters from organic synthesis production lines and oil refineries [18].

The processes of biodegradation of aromatic compounds can be characterized by Haldane equation: $\mu = \mu_{max}S/[k_s+S+(S^2/k_i)]$ which is frequently used to describe such type of degradation [6]. The value of k_i is equal to the lowest concentration of the inhibitor which causes a decrease in microbial growth rate.

The present paper deals with processes of degradation and utilization of monohydroxyl derivatives of phenol (resorcinol, catechol and hydroquinone), as well as some of the most toxic aromatic pollutants of the environment like 2,6 - dinitrophenol, α-methyl styrene and acetophenone. The basic kinetic parameters for the biodegradation of the listed above compounds are reported.

2. Materials and methods

2.1. Microorganisms and growth conditions

The strain used trough all the experiments *Trichosporon cutaneum* R57 is registered in NBIMCC, N2414/94, Bulgaria.

The inoculum of *Trichosporon cutaneum* R57 strain was grown in the liquid medium containing 10 g/l yeast extract and 20 g/l peptone (YEP) and 0.5 g/l phenol. The cultivation of all the batch experiments was carried out in 50ml YNB w/o AA(6.7g/l), Fluka. The initial OD_{610} values were adjusted to 0.135 ± 0.02. The pH value was 6.0. The various concentrations of used carbon sources were added in the medium as sole source of carbon and energy. The cultivation was performed on a New Brunswick rotary shaker at 28-30^{0}C and 200 rev/min.

2.2. Analytical methods

Cell density was monitored spectrophotometrically (LKB UV-Vis Ultraspec 1000) at λ=610nm.

The cell free supernatants were analyzed by HPLC performed on a reverse phase C_{18} column (Licrosorb Rp18, Perkin-Elmer) with methanol-water (50:50) mobile phase using a UV detector set at 220nm.

3. Results and discussion

3.1. Growth and degradation of hydroxyl substituted phenols by *Trichosporon cutaneum* R57

Hydroxyl aromatic compounds like resorcinol, catechol and hydroquinone are widely used for production of various chemical reagents. These compounds can be utilized by many organisms at low concentration, but at high concentrations they can also act as toxic growth inhibitors.

In our earlier experiments we have cultivated *Trichosporon cutaneum* R57 strain in various culture media including different minimal or synthetic media [19]. We choose to carry out our cultivation experiments in YNB w/o AA medium. This medium gives an opportunity to investigate the yeast capacity to assimilate different compounds as sole carbon sources [20]. It has been reported that *Trichosporon cutaneum* R57 can grow in an YNB medium containing 0.8 g/l resorcinol as the only source of carbon and energy [19]. In the present study the time for resorcinol degradation obtained was much shorter which is due to the inoculum pre-incubation in a phenol containing medium as distinct from our earlier experiments. The growth of the strain and degradation dynamics of resorcinol, catechol and hydroquinone in different concentrations varying from 0.1 up to 1.6 g/l were studied. On Fig.1 are shown the curves of specific growth rates demonstrating the influence of initial substrate concentrations (resorcinol – 1.6 g/l; catechol – 1.3 g/l; hydroquinone – 1.2 g/l) on biodegradation kinetics. The curves character showed an inhibitory effect on microbial growth of the initial hydroquinone, resorcinol and catechol concentrations greater than 0.6 g/l.

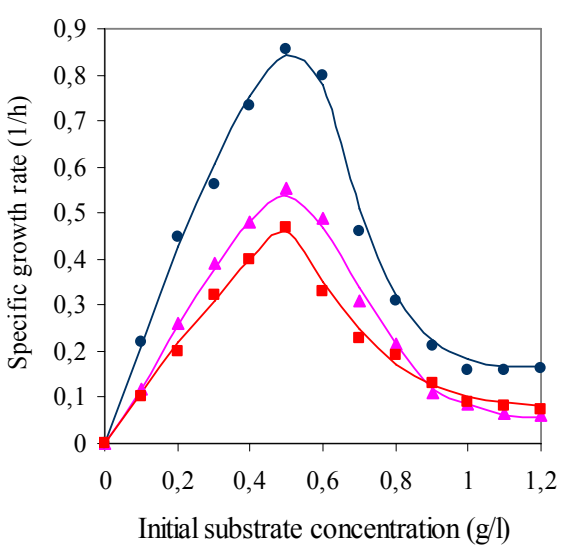

Fig. 1. Effect of different initial concentrations of (●) hydroquinone, (■) catechol, (▲) resorcinol on specific growth rate (μ) of *Trichosporon cutaneum* R57.

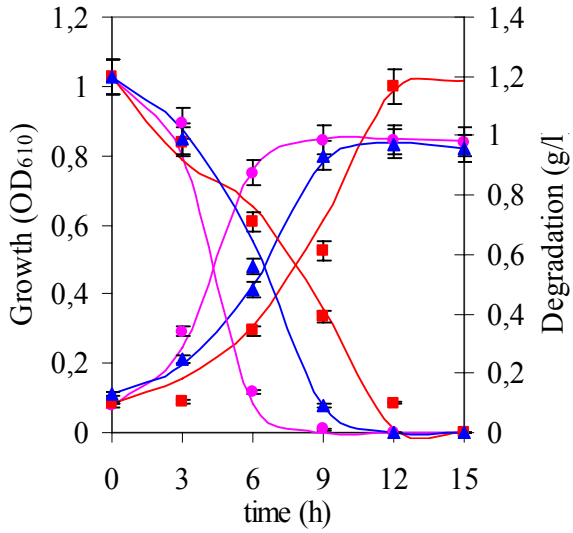

Fig. 2. Experimental data (symbols) and model profiles (curves) for: growth of *Trichosporon cutaneum* R57 on (●) hydroquinone, (■) catechol, (▲) resorcinol and decrease of growth substrate from the medium.

Fig.2 shows the curves of growth and degradation obtained as results from strain cultivation in YNB w/o AA medium containing separately the three compounds mentioned above in concentration of 1.2 g/l. The demonstrated concentration indicated very well the high ability of the investigated strain to utilize these compounds and allowed a good parallel of the growth dynamics to be done.

Gaal and Neujahr [21] have studied hydroxyl phenols degradation by *Trichosporon cutaneum* yeast. Krug et. al. [22] have investigated the utilisation of hydroxyl phenols in concentrations of 0.15 g/l by *Candida tropicalis* HP 15. Kurtz and Crow [7] have described a strain of *Candida maltosa* able to degrade 5 mM resorcinol. The obtained in this research degradation activity of *T. cutaneum* R57 was much higher than reported in the literature data. The time necessary for complete degradation of 1.2 g/l of these phenol derivatives was found to be within 9-12h including short adaptation phase (1-3h).

Table 1. Biokinetic parameter values for toxic substrates degradation by *Trichosporon cutaneum* R 57

Compound	Haldene's model		
	μ_{max} (h^{-1})	Ki (g/l)	Ks (g/l)
resorcinol	0.55	0.580	0.22
hydroquinone	0.85	0.600	0.15
catechol	0.47	0.550	0.23
acetophenone	0.26	0.15	0.10
α-methylstyrene	0.45	0.25	0.20
2,6 – dinitophenol	0.48	0.13	0.11

The inhibition coefficients (resorcinol - k_i=0.58 g/l; catechol - k_i=0.55 g/l; hydroquinone - k_i=0.6 g/l) were calculated according to the Haldane-kinetics. The values of the process parameters (μ_{max}, k_s, k_i and k) were derived by using a nonlinear least – squares regression analysis of the *Statistics* toolbox of *Matlab* 6.1. In all cases, the maximum deviations were approximately 13 % and the coefficients of correlations R^2 were found to be more than 0.98, which indicated the model results agreed with the experimental data very well. The obtained substrate affinity constants (k_s) for studied hydroxyl phenols and maximum specific growth rates (μ_{max}) were also comparable to each other, within an interval from 0.15 to 0.22g/l and from 0.5 to 0.8h^{-1}, respectively (Table 1).

3.2. Growth and degradation of 2,6 dinitrophenol, α-methylstyrene and acetophenone by *Trichosporon cutaneum* R57

Nitro-aromatic compounds are widely spread contaminants generated by industrial production of insecticides, herbicides, fungicides and explosives. These compounds accumulate in the environment due to their high toxicity and very good water solubility. The acetophenone and *α*-methylstyrene are among the most toxic aromatic components of the wastewaters from oil refineries and chemical industry**.** Waste from the production of synthetic rubber can contain *α*-methylstyrene, which is toxic for both humans and the biological systems of rivers.

There is not many data published about biodegradation of these highly toxic aromatics. The ability of some microorganisms to utilize such compounds as sole source of carbon and energy is just mentioned in most of them. [23, 24]. Strains of the genera *Nocardia*, *Arthrobacter* and *Trichosporon* which use acetophenone as a sole source of carbon and energy were described [25, 26]. Microorganisms degrading *α*-methylstyrene have been the subject of investigations [27,28].

Earlier we reported the ability of the *Trichosporon cutaneum* R57 to grow on a medium supplemented with high concentrations of 2,6 – dinitrophenol (0.7 g/l), α-methyl styrene (0.5 g/l) and acetophenone (0.5 g/l) but the growth dynamics was not investigated. Each of these compounds was comprised separately as sole carbon and energy source in the medium YNB w/o AA.

Fig.3 shows the curves of specific growth rates which present the influence of initial concentrations of 2,6 – dinitrophenol, α-methyl styrene and acetophenone on growth kinetics of *Trichosporon cutaneum* R57 strain. The established values of specific growth rates demonstrate an inhibitory effect of certain substrates concentrations. The maximum specific growth rates were obtained as: 0.26 h^{-1} at an initial concentration of acetophenone of 0.125 g/l; 0.45 h^{-1} at an initial α-methyl styrene concentration of 0.225 g/l and 0.48 h^{-1} at an initial concentration of 2,6 – dinitrophenol of 0.125 g/l. Higher concentrations of investigated substrates show an inhibitory effect on microbial growth.

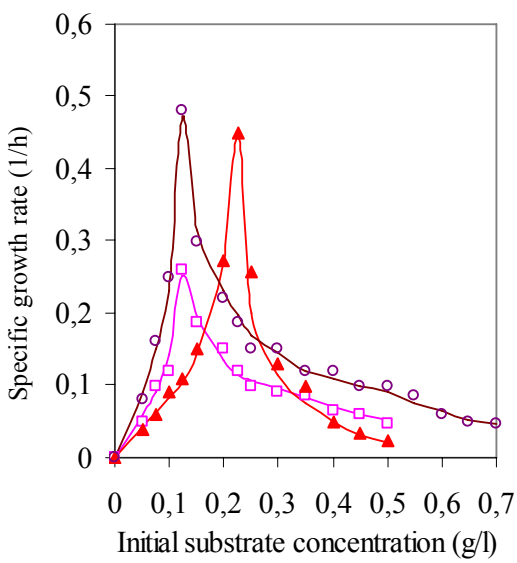

Fig. 3. Effect of different initial concentrations of (▲) α-methylstyrene, (○) 2,6-dinitrophenol, (□) acetophenone on specific growth rate (μ) of *Trichosporon cutaneum* R57.

In the present study we demonstrate the growth and degradation capacity obtained by measuring the decrease of cited above aromatics in the process of *Trichosporon cutaneum* R57 cultivation (Fig.4). Complete degradation of 0.5 g/l of any of the investigated compounds was observed after 18 – 24 hours.

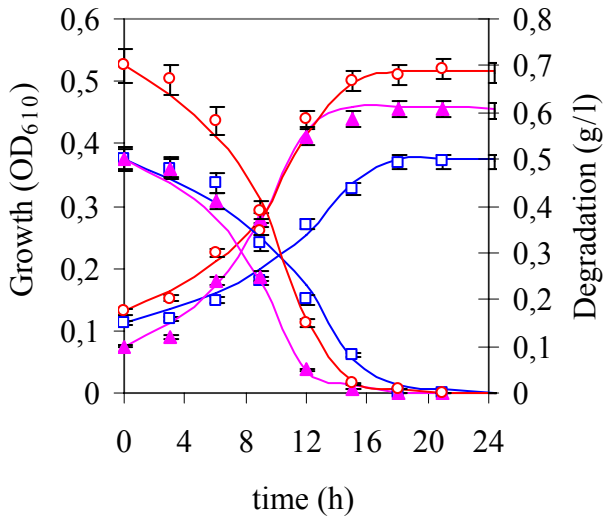

Fig. 4. Experimental data (symbols) and model profiles (curves) for: growth of Trichosporon cutaneum R57 on (▲) α-methylstyrene, (○) 2,6 dinitrophenol, (□) acetophenone and decrease of growth substrate from the medium.

The data about development and degradation characteristics of the investigated strain were processed according to the Haldane-kinetics. The values of the growth kinetics parameters obtained for the investigated compounds are given in Table 1. We should point out the significant difference between inhibitory coefficients for both groups investigated aromatic compounds. The lower values of k_i as a whole for 2,6–dinitrophenol, α-methylfstyrene and acetophenone are an expression of their high toxic effect on *Trichosporon cutaneum* R57 strain. Nevertheless, the established values of k_s constants are comparable to those which are characteristic of monohydroxyl phenols.

Acknowledgments The support by NCSR of the Bulgarian Ministry of Education and Science under project K 1205/02 is gratefully acknowledge.

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Biodegradation of exogenous DNA by bio-products used in domestic sewage treatment

Hanna Kruszewska[*,1], Aleksandra Misicka[2], Stefan Tyski[1,3]

[1] National Medicines Institute, 30/34 Chełmska, 00-725 Warsaw, Poland
[2] University of Warsaw, Department of Chemistry, 1 Pasteura, 02-093, Warsaw, Poland
[3] Medical University of Warsaw, Department of Pharmaceutical Microbiology, 3 Oczki, 02-007 Warsaw, Poland

[*]Corresponding author e-mail: hanna@il.waw.pl

Exogenous DNA may participate in a natural genetic transformation of bacteria. This process may lead to the acquisition of new features, such as resistance to antibiotics. Four bio-products used in domestic sewage treatment were examined in order to check their ability to degrade exogenous DNA and DNA nucleosides derivative antiviral drugs (AZT and acyclovir). Three bio-products (*Bio 7 CHOC-granulate*, *Bio 7 CHOC-capsules* and *Septifos*) were able to degrade DNA. After 240 h of treatment, nucleic bases or their derivatives were found in the reaction mixture. Neither nucleic bases nor their derivatives nor peak absorption at 254 nm were found in the medium after 480 h of reaction. The ability to degrade DNA to CO_2 and NH_3 or N_2 is not a common feature among the microorganisms. This activity was not mentioned in advertisement materials. Interestingly, only one bio-product tested (*Septifos*) was able to degrade AZT in anaerobic environment. Acyclovir was resistant to degradation by any bio-products.

Keywords Bio 7; Septifos; AZT; acyclovir

1. Introduction

Nowadays small domestic sewage treatment plants became popular way of domestic waste water treatment. Waste collector connected to house sewerage is usually placed underground near the house. Proper caring for the receiver requires frequent addition of waste decomposing bio-products (usually containing mixture of bacteria and/or fungi) degrading lipids, proteins and carbohydrates. Although the advertising materials attached to the bio-products claim that purified sewage can be used for soil irrigation [1], little is known about the ability of the bio-products to degrade DNA.

The amount of free microbial DNA derived from human environment in biological sewage treatment plants or domestic sewage treatment is significant [2]. If not degraded, even small amount of DNA originating from the lysis of bacterial, plant or animal cells or human/animal viruses, may participate in a natural genetic transformation of bacteria that encompasses an active uptake of free (extracellular) DNA and a heritable incorporation of the genetic information into bacterial genome [3]. This process may lead to the acquisition of new features, such as resistance to antibiotics [4,5]. Free DNA content present in environment varies depending on the type of environment (soil, rivers, sewage etc.) [3] and microorganisms living in the environment. If exonucleases secreting microorganisms are present in the environment, DNA is quickly degraded [6]. However, DNA adsorbed on the surface of sediment minerals is relatively resistant to degradation by exonucleases and thereby maintains its transforming activity for naturally competent bacterial cells [7-10].

In addition, DNA nucleosides derivative drugs that constitute an essential part of modern human and veterinary medicine might be present in domestic sewage. Whereas some of these drugs are metabolized in the human body, others, such as azidovudine (AZT) and acyclovir, are excreted unchanged. Acyclovir is excreted unchanged from organism in urine, only up to 14% is metabolized to 9-carboxymethoxymethylguanine [11]. AZT is excreted in urine unchanged (10-20%) or as 5'-O-ether glucuronide derivative (50-80%) [12]. If drug residues have not been completely removed during passage through a sewage treatment plant, than enter the aquatic environment. Some studies have shown that a multitude of drugs are present in aquatic systems [13-15].
The aim of this study was to obtain the information whether the commercial biological products (mixtures of microorganisms) used in domestic sewage treatment are able to degrade DNA and pharmaceutical substances: AZT and acyclovir.

2. Materials and methods

2.1 Materials

2.1.1 Commercial bio-products

Four commercial bio-products used in this study, Bio 7 CHOC-granulate and Bio 7 CHOC-capsules (Laoratoria Ecogene), Microbe-lift (Ecological Laboratories INC, USA) and Septifos (Buhler-Kiwi France), were supplied by DANEKO (Warsaw, Poland). All bio-products were used in concentration recommended by the manufacturer for purification sewages in domestic sewage treatment plants.

2.1.2 Chemicals

High molecular weight DNA from salmon milt, DNA nucleotides (dAMP, dCMP, dGMP, dTMP), respective nucleosides, bases and derivatives were obtained from Pharma Waldhof GmbH (Dusseldorf, Germany).
AZT (azidovudine): - 3'-azydo-3'-deoxythymidine was obtained from Institute of Industrial Chemistry (Warsaw, Poland).

Acyclovir: 9-[(2-hydroxyetoxy)-metylo]-guanine was bought in Union Quimico Farmaceutica-Barcelona (Spain).

2.1.3 Method of degradation

The suitable amount of bio-product to obtain the concentration recommended by the manufacturer was resuspended in 0.08 M phosphate buffer (pH 7.0 ± 0.2) supplemented with 0.5% glucose to obtain 50 ml of suspension. The reaction was started by addition of 50 mg of DNA or 25 mg pharmaceutical substance to the 50 ml of bio-product suspension. The mixture was incubated at 30°C in a rotary shaker at 100 rpm and samples were taken in indicated time intervals.

2.1.4 Methods of analysis

The filtrated sample (20 µl) diluted 10 times in a mobile phase was injected into Hyprsil 120 ODS column (25 cm, 4.6 mm x 5 µm) and analysis was performed on HPLC apparatus with spectrophotometric detector UV/VIS (λ=254 nm). An aqueous buffer (0.02 M NaH_2PO_4, pH 4) with acetonitrile (3.8 %) was used as a mobile phase (flow rate 1 ml/min). The quantitive determination of reaction products were performed by the external standard calibration method (Table. 2).

Bacterial colony forming units were estimated by plate-count method on TSA (Tryptic Soy Agar) and Sabouraud agar. Anaerobic condition was created by Genbox anaer (BioMerieoux).

3. Results and discussion

Four bio-products (*Bio 7 CHOC-granulate*, *Bio 7 CHOC-capsules*, *Microbe-lift* and *Septifos*) were analysed for their ability to degrade an exogenous DNA. Three bio-products were able to degrade DNA (1 mg/ml): *Bio 7 CHOC-granulate*, *Bio 7 CHOC-capsules* and *Septifos*. DNA in concentration 1 mg/ml did not influence growth of any tested microorganisms in bio-products. The highest concentration of DNA, which did not influence the growth of microorganisms was 3 mg/ml. Slight inhibition of bacterial growth was noticed in concentration of 5 mg/ml DNA. It was already reported that this concentration of DNA inhibited also growth of *S. maltophilia* [16].

The highest rate of degradation of high molecular weight DNA to nucleic bases and their derivatives was observed for *Bio 7 CHOC-capsules* after 144 h of treatment. This result is similar to that found in case of DNA degradation by *S. matophilia* and *M. butyricum* [16] (Table 1).

Table 1. Time of DNA degradation by bio-products and bacterial strains

Bio-product or bacterial strain	Degradation time (h)
Septifos	192
Bio 7 CHOC-capsules	144
Bio 7 CHOC-granulate	240
Mycobacterium butyricum [16]	240
Stenotrophomonas maltophilia [16]	120

Degradation of DNA by the bio-products resulted in accumulation of thymine, hypoxanthine and uracyl in reaction medium (Table 2). Interestingly, neither nucleic bases nor their derivatives nor any other compounds with peak absorption at 254 nm were found in medium after 480 h of reaction. We conclude that above mentioned bio-products are capable to completely degrade DNA, likely to CO_2 and NH_3 or N_2. The ability to degrade DNA to CO_2 and NH_3 or N_2 is not a common feature of microorganisms, thus the ability of bio-products used in domestic sewage treatment to carry out the complete degradation of DNA is beneficial, as the amount of DNA from bacterial lysates is high in this type of waste. This activity was not mentioned in advertisement materials of the tested bio-products. Neither *S. maltophilia* nor *M. butyricum*, previously tested for DNA degradation ability, were capable of complete degrading DNA. Both strains were able to degrade DNA to nucleic bases and their derivatives, which accumulated in the reaction medium [16].

Table 2. Products of DNA degradation by bio-products after time of reaction showed in Table 1

Bio-product or bacterial strain	Degradation products (μg/ml)						
	Adenine	Thymine	Hypoxan-thine	Xanthine	Cytosine	Uracil	Guanine
Septifos	-	28	34	24	-	19	-
Bio 7 CHOC-capsules	7	34	93	-	-	21	-
Bio 7 CHOC-granulate	5	45	72	-	13	32	24
M. butyricum [a] [16]	-	-	11	24	-	-	-
S. maltophilia [a] [16]	-	32	29	30	-	33	-

[a] measured after 480 h of reaction

The second aim of this study was to check the ability of the bio-products to degrade DNA bases derivatives - pharmaceutical substances: AZT and acyclovir. Both substances in concentration 0,5 mg/ml did not significantly influenced growth of the bio-products. AZT was degraded only by one bio-product - *Septifos*. The degradation took place only in anaerobic condition and resulted in 5 undetermined compounds. Acyclovir was resistant to degradation by any bio-products in aerobic or anaerobic conditions. Only limited number of bacterial strain (*Stenotrophomonas matophilia, Escherichia coli, Pseudomonas aeruginosa*, *Brevundimonas diminuta*, *Lactobacillus acidophilus*) was checked for their ability to degrade the AZT. This compound was resistant to degradation by bacteria except *S. matophilia* and *B. diminuta* which hydroxylated AZT to 3'-azido-3'-deoxy- -ribosylthymine [17,18].

4. Conclusions

Bio 7 CHOC-granulate, *Bio 7 CHOC-capsules* and *Septifos* completely degraded DNA. This advantageous activity was not mentioned in advertisement materials of the bio-products.

Only one bio-product (*Septifos*) was able to degrade AZT in anaerobic condition to 5 undetermined compounds.

Acyclovir was resistant to degradation by any bio-products tested in aerobic or anaerobic conditions.

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Biotechnological Potential of *Phanerochaete chrysosporium* UCP 963 and *Cunninghamella elegans* UCP 596 in the Copper and Zinc Removal

M. A. Moraes Filho[1,2], M. C. Freitas Silva[1,2]; T. C. M. Stamford[2]; K. Okada[2,3]; G. M. Campos-Takaki[2,4,*]

[1] Doutorado em Ciências Biológicas, UFPE/Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235 - Cidade Universitária, Recife - PE - CEP: 50670-901, Brazil.
[2] NPCIAMB/Núcleo de Pesquisas em Ciências Ambientais, Universidade Catíolica de Pernambuco, Brazil.
[3] Centro de Ciências Biológicas e Saúde, Universidade Católica de Pernambuco, Brazil.
[4] Centro de Ciências e Tecnologia, Universidade Católica de Pernambuco, Rua Nunes Machado nº42, CEP: 50050-590, Recife, PE, Brazil.

[*]Corresponding author: e-mail: takaki@unicap.br, Phone: +558121264017

Inquiries comparatively was carried out on the ability of biosorption of copper and zinc for the biomass of *Phanerochaete chrysosporium* UCP 963 and *Cunninghamella elegans* UCP 596. The strain of *C. elegans* showed ability to removal of heavy metals in the concentration of 4 mM with incomes of 55% of removal of copper, and 51% of zinc, respectively, and in the concentration of 6 mM zinc was removed 53% and copper 57%, all the treatment using 120 mg of the biomass. The inactivated biomass of *P. chrysosporium* was more efficient in the copper removal in the concentration of 6 mM with results of 45% of removal and in both zinc concentrations (4 and 6 mM) it presented a sorption of 59-63 %, respectively, during 480 minutes. The results demonstrate that inactivated biomass and/or live biomass of *P. chrysosporium* and *C. elegans* presents ability of removal of copper and zinc.

Keywords Biosorption; Copper; Zinc; *Phanerochaete chrysosporium*; *Cunninghamella elegans.*

Investigações foram realizadas sobre a habilidade de biossorção de cobre e zinco pela biomassa de *Phanerochaete chrysosporium* UCP 963 e *Cunninghamella elegans* UCP 596. A amostra de *C. elegans* demonstrou habilidade de remoção dos metais pesados na concentração de 4 mM com rendimentos de 55% de remoção para o cobre, 51% de remoção de zinco, e na concentração de 6 mM zinco removeu 53% e cobre 57%, todos os tratamentos foi utilizado 120 mg da biomassa. A biomassa inativada de *P. chrysosporium* foi mais eficiente na remoção de cobre na concentração de 6 mM com resultados de 45% de remoção e em ambas concentrações de zinco (4 e 6 mM) apresentando uma sorção de 59-63 %, respectivamente, durante 480 minutos. Os resultados demonstram que tanto a utilização da biomassa inativada e/ou viva de *P. chrysosporium* e *C. elegans* apresentam habilidade no processo de remoção de cobre e zinco.

Palavras-chave Biossorção; Cobre; Zinco; *Phanerochaete chrysosporium*; *Cunninghamella elegans*.

1. Introduction

Heavy-metal pollution represents an important environmental issue due to the toxic effects of metals, and their accumulation throughout the food chain, leads to serious ecological and health problems. Metal remediation through common physicochemical techniques is expensive and unsuitable in case of voluminous effluents containing complexing organic matter and low metal contamination [9].

It is well recognized that microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms. These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals, also in cleaning up or remediating metal-contaminated environments [13].

The mechanism by which microorganisms remove metals from solutions are: (i) extracellular accumulation/precipitation; (ii) cell-surface sorption or complexation; and (iii) intracellular accumulation. Among these mechanisms, extracellular accumulation/precipitation may be facilitated by using viable microorganisms, cell-surface sorption or complexation can occur with alive or dead microorganisms, while intracellular accumulation requires microbial activity [6].

The use of dead cells offers the following advantages: (a) metal removal system is not subjected to metal toxicity limitations; (b) no requirements for growth media and nutrients; (c) adsorbed metal ions can be easily

desorbed and biomass can be reused; and (d) the dead biomass based adsorption system can be subjected to conventional theories and mathematical models already in use for traditional adsorption systems [8].

The anthropogenic sources of heavy metals include wastes from the electroplating and metal finishing industries, metallurgical industries, tannery operations, chemical manufacturing, mine drainage, battery manufacturing, leather tanning industries, fertilizer industries, pigment manufacturing industries, leachates from land fills and contaminated ground water from hazardous waste sites [6].

In general, treatment of effluents contaminated with metals involves physicochemical process of flocculation and/or precipitation, electrolysis, crystallization and adsorption. However, these processes can be expensive or lead to production of new contaminants. Thus, they need for more economic and practical technologies for the metal removal from effluents has resulted in the search for new methods that may be useful in reducing the levels of metals or even in recovering them from industrial effluents [5].

Metals occur naturally in the environment and are present in rocks, soil, plants, and animals. Metals occur in different forms: as ions dissolved in water, as vapors, or as salts or minerals in rock, sand, and soil. They can also be bound in organic or inorganic molecules, or attached to particles in the air. Both natural and anthropogenic processes emit metals into air and water [3].

Heavy metals in high concentrations are considered toxic the human been, therefore they can affect the nerves system, liver and bones and they block functional groups of vital enzymes. Amongst the biosorbent most common are marine algae, bacteria, yeasts, fungi and waste mycelia from the fermentations and food- industry [2].

The filamentous fungi are known by possessing an accumulation potential, or either, a gamma of cátions of metal in water solutions as, has covered, Uranium, Nickel, and Cobalt. Living microbial cells usually exhibit two distinct phases of metal accumulation. The first involves metal adsorption around the cell envelop and is commonly considered metabolism-independent process (biosorption). Second it is exclusively dependent on cell metabolism and evolves active translocation of metals by microorganisms into the cell (bioaccumulation). Cations exchange has been pointed as the biggest mechanism of metals adsorption under the biosorbents produced for biomass [5, 12].

Considering the role of fungi, in xenobiotic metabolization/bioremediation and biosorption, this work has as objective to evaluate the ability of the inactivated or live biomass of the *Phanerochaete chrysosporium* and *Cunninghamella elegans* in the process of heavy metals removal.

2. Material and Methods

2.1. Microorganisms

Two strains were used *Phanerochaete chrysosporium* UCP 963 and *Cunninghamella elegans* UCP 596, kindly obtained from Cultures Bank of Universidade Católica de Pernambuco, maintained in media potato dextrose agar at 4°C.

2.2. Spore production

The sporangiole production by *C. elegans* was used the yeast malt agar (YMA), with the following composition in $g.L^{-1}$ of distilled water: yeast extract, 3,0; peptone, 5,0; malt extract, 3,0; glucose, 10,0; agar 15,0; pH 5.0. The potato dextrose broth media (VETEC) was used to spore production by *P. chrysosporium*. The samples of *P. chrysosporium* and *C. elegans* were inoculated in Petri plates with 9 cm of diameter, contend media specific for sporulation and incubates during 6 days at 28°C. After this period, the spores were collected, with humidified swabs in distilled water, being determined the concentration of spores in Neubauer chamber. Aliquot of 1 mL of the suspension of 10^7 spores/mL were used as pre-cultivation.

2.3. Biomass Production and Inactivation

The biomass production by *C. elegans* was used malt extract 3%, and potato dextrose broth (VETEC) for *P. chrysosporium*. About 5 g of the lyophilized mycelia of *P. chrysosporium* and *C. elegans* was immersed in 20 mL of deionizated sterile water and inactivated in for 15 minutes, at temperature of 121°C.

2.4. Heavy Metals Removal and Determination

The removal of heavy metals was carried using different amounts of biomass (120 mg and 240 mg), fresh and inactivated, added of 65 mL of the zinc solution (ZnSO4.7H2O) and copper (CuSO4.5H2O) solution (4 and 6 mM), in Erlenmeyer's flasks, and pH 5,0, under agitation 150 rpm at 28 ºC , during 480 minutes. Aliquot was

removed in the intervals of 30, 120 and 480 minutes prefaced a total of 8 experimental assays. The concentration of metals was analyzed using CHEMetrics Vacu-vials kit for Copper and Zinc and for spectrophotometer of atomic absorption. The heavy metals biosorption ability by biomass, inactivated and fresh, from *P. chrysosporium* and *C. elegans*, were determined by the amount of heavy metals in aqueous solution, before and after the contact with biomass and was express in accordance with the following mathematical expression described by Jeon *et al*., 2002:

$$Q = CiVi - CfVf / m,$$

where Q is the capacity of sorption of the metal heavy ($mg.g^{-1}$ of biomass), Ci are the initial concentration of the metal heavy ($mg\ L^{-1}$), Cf are the final concentration of the metal ($mg\ L^{-1}$), Vf are the final volume (L^{-1}), Vi are the initial volume (L^{-1}) and "m" is the initial biomass in (g).

3. Results and Discussion

3.1. Copper biosorption by *P. chrysosporium* and *C. elegans*

The Table 1 shows the capacity of copper biosorption by *C. elegans* biomass in conditions established, the results indicate variation between 33-171 $mg.g^{-1}$. The best results were demonstrated with 120 mg of live biosorbent, entered in contact with 4 mM of copper solution, occurring best removal in 120 minutes, around 104.96 $mg.g^{-1}$ of biomass (55% of removal). In 6 mM of copper solution was observed the removal of 171.60 $mg.g^{1}$ (57% of removal) of the metal in the 120 mg conditions of live biomass, after 2 hours of agitation.

Table 1 Biosorption capacity by inativacted and live biomass of *C. elegans* UCP 596, under concentrations of 4 and 6 mM of copper.

COPPER CONCENTRATION				
Biomass	**Initial ($mg.L^{-1}$)**	**Copper (Biomass. $mg.g^{-1}$)**		
		30 min	**120 min**	**480 min**
Live (120 mg)	347.0	7.13	104.96	13.06
Live (240 mg)		11.51	33.63	9.20
Inactivated (120 mg)		34.85	78.75	35.41
Inactivated (240 mg)		17.40	38.46	19.36
Live (120 mg)	550.0	47.95	171.60	39.74
Live (240 mg)		30.79	54.16	32.53
Inactivated (120 mg)		74.45	139.86	88.89
Inactivated (240 mg)		25.67	66.05	39.33

Table 2 Biosorption capacity by inativacted and live biomass of *P. chrysosporium* UCP 963, under concentrations of 4 and 6 mM copper removal.

COPPER CONCENTRATION				
Biomass	**Initial ($mg.L^{-1}$)**	**Copper (Biomass. $mg.g^{-1}$)**		
		30 min	**120 min**	**480 min**
Live (120 mg)	347.0	4.91	39.15	65.25
Live (240 mg)		6.87	23.27	33.90
Inactivated (120 mg)		14.93	38.99	75.71
Inactivated (240 mg)		17.09	4.14	54.24
Live (120 mg)	550.0	12.89	77.19	122.78
Live (240 mg)		13.90	49.46	61.84
Inactivated (120 mg)		47.04	59.56	134.18
Inactivated (240 mg)		35.27	39.15	88.54

Table 2 presents the occurrence of a variation in the capacity of copper biosorption by *P. chrysosporium* around 33-134 $mg.g^{-1}$ of biomass. In table 2 can see that 4 mM concentration of copper had similar results, both live and inactivated, 120 mg biomass presents values around 65-75 $mg.g^{-1}$ (35-40% of removal) for 480 minutes of contact.

These results agrees with studies carried by Baik *et al.* 2002, where the authors had demonstrated to copper removals equivalent 50 and 110 $mg.g^{-1}$ for *Aspergillus niger* and *Mucor rouxii*, respectively. Siegel *et al.*,1983, verified capacity of copper biosorption of 80 $mg.g^{-1}$ of biomass by *Penicillium notatum*. Volesk *et al.* 1993, using *Saccharomyces cerevisiae* got an income of 71 $mg.g^{-1}$ of biosorption.

3.2. Zinc biosorption by *P. chrysosporium* and *C. elegans*

The results presented in Table 3 shows a variation in the biosorption capacity of zinc by *C. elegans* around 42-129 $mg.g^{-1}$ of biomass. Verified that in the concentration of 4 mM of zinc the biosorption incomes, by 120 mg of live and inactivated biomass, was more efficient, with 94,44 $mg.g^{-1}$ and 90,47 $mg.g^{-1}$ (51 and 47% of removal), respectively, in 480 minutes. Similar results can be observed in the metal concentration of 6 mM, that was around 129 mg.g-1 (53% of removal) for 120 mg of inactivated biomass, for 120 minutes.

Table 3 Biosorption capacity by inativacvted and live biomass of *C. elegans* UCP 596, under concentrations of 4 and 6 mM of zinc

ZINC CONCENTRATION				
Biomass	**Initial ($mg.L^{-1}$)**	**Zinc (biomass. $mg.g^{-1}$)**		
		30 min	**120 min**	**480 min**
Live (120 mg)	261.6	45.73	69.39	94.44
Live (240 mg)		28.12	40.76	51.19
Inactivated (120 mg)		42.86	65.34	90.47
Inactivated (240 mg)		22.39	11.02	42.26
Live (120 mg)	392.4	0.16	98.97	97.10
Live (240 mg)		37.73	63.23	68.41
Inactivated (120 mg)		60.16	129.71	124.97
Inactivated (240 mg)		36.77	53.53	65.76

Table 4 Biosorption capacity by inativacvted and live biomass of *P. chrysosporium* UCP 963, under concentrations of 4 and 6 mM of zinc.

ZINC CONCENTRATION				
Biomass	**Initial ($mg.L^{-1}$)**	**Zinc (biomass $mg.g^{-1}$)**		
		30 min	**120 min**	**480 min**
Live (120 mg)	261.6	18.01	66.15	58.04
Live (240 mg)		0	34.69	33.65
Inactivated (120 mg)		18.01	23.28	101.72
Inactivated (240 mg)		19.04	27.41	27.36
Live (120 mg)	392.4	2.80	90.88	107.69
Live (240 mg)		0	18.34	51.86
Inactivated (120 mg)		0	56.90	101.08
Inactivated (240 mg)		0	73.34	53.18

The sorption results represented in Table 4 demonstrates that the capacity of sorption of zinc by *P. chrysosporium* varied at minimum of 51 $mg.g^{-1}$ to the maximum of 107 $mg.g^{-1}$, in 480 minutes of biomass contact with the metal solution. The biggest income of biosorbent was observed in the 6 mM of metal concentration, using 240 mg of inactivated biomass, that was of 73,34 $mg.g^{-1}$ (63% of removal). Similar capacity was observed in the concentration of 4 mM, that was of 101,72 $mg.g^{-1}$ of biomass.

Inferior results gotten was demonstrated by Arica *et al.,* 2003, that they had used the biomass live and inactivated by *P. chrysosporium* getting incomes 37 to 47 $mg.g^{-1}$, respectively. Ozer *et al.,* 1997, using the biomass of *Rhizopus arrhizus* got resulted lesser to the presented here, with 62 $mg.g^{-1}$ of removal capacity. Yan & Viraraghavan 2003, demonstrated that the removal capacity for the biomass by *Mucor rouxii* was 44.21 $mg.g^{-1}$. Fourest *et al.,* 1994, showed results for *R. arrhizus*, *Mucor miehei* and *Penicillium chrysogenum*, as incomes of 35, 32 and 22 $mg.g^{-1}$ of biomass, respectively.

Aknowledgements The authors are grateful to CNPq, CAPES, and FACEPE.

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Cadmium toxicity on *Cunninghamella elegans*: Ultrastructural damage and actin cytoskeleton alterations

M.A.B. Lima [1,3,4], P.M. Souza [1,3], P.H.C.Marinho[1,2], T.C.M. Stamford [1,4], A.E. Nascimento[1], and G.M. Campos-Takaki [1,2,3,*]

[1] Nucleus of Research in Environmental Sciences - Catholic University of Pernambuco, Recife, PE, Brazil
[2] Biological Sciences Post-Graduation Programme - Federal University of Pernambuco, Recife, PE, Brazil
[3] Fungi Biology Post-Graduation Programme - Federal University of Pernambuco, Recife, PE, Brazil
[4] Integrated College of Patos, Patos, PB, Brazil

In this study *Cunninghamella elegans* was studied in relation to its structural behavior in the presence of cadmium. The organism was cultured in cadmium concentrations of 5.62mg/L, 11.24mg/L, and 22.10mg/L and was submitted to fluorescence and electronic microscopy, using a routine technique and ruthenium red cytochemistry. The fluorescence study revealed differences in the actin cytoskeleton pattern related to form, distribution and arrangement. The transmission electron microscopy showed fine structure alterations in the cell electron-density, cytoplasm vacuolization, electron-dense inclusions and granulations in the cytoplasm. The ruthenium red cytochemistry revealed sites of reaction products on the cell surface and in the cytoplasm. These results suggest, for the first time, the cadmium effects on the cellular fine structure and in the actin cytoskeleton of zygomycetes *C. elegans*.

Keywords Toxicity, *Cunninghamella elegans*; Cadmium; Ultrastructure; Actin Cytoskeleton

1. Introduction

Microbial cells are constantly exposed to stressful situations and the ability to overcome those stresses is essential for their survival. The microorganisms are able to bind metals, and when they accumulate, this occurs by means of physiological mechanisms. Bacteria, fungi, actinomycetes and algae are among the effective biological machines to sequestrate metallic ions, and have been used to remove metals from industrial and domestic effluents. Metal–microbe interactions have an important role in several biotechnological applications, including biomineralization, bioremediation, bioleaching and microbial corrosion, besides having attracted growing attention in recent years [1-4].

There are substantial data related to microbial behavior under stress situations induced by heavy metals, which could be associated with enzyme activity, cellular count, biomass carbon and nitrogen content. The mechanisms responsible for cellular resistance/tolerance toward metals are directly related to physiological and metabolic pathways. The cellular surface is the main structure associated with interactions with the environment and its characteristics [5-8].

2. Materials and Methods

2.1 Microorganisms and Culture Conditions

The 46109 IFM strain of *Cunninghamella elegans* used in this study was kindly supplied by the Culture Collection of the Research Center for Pathogenic Fungi and Microbial Toxicosis, Chiba University- Japan. A total of $1x10^5$ spores/ mL of *C. elegans* were collected and transferred to 125 mL Erlenmeyer flasks containing 50 mL of Synthetic Medium for Mucorales (SMM) described by Hesseltine and Anderson [9] supplemented with cadmium in the concentrations of 5.62mg/L; 11.24mg/L and 22.10mg/L (monohydrate chloride salts). The control cultures were grown in the original medium, without cadmium. The cultures were incubated for 3 days, at 28°C, at 250 hz. All samples were prepared in three replicas.

2.2 Transmission Electron Microscopy (TEM)

2.2.1 Routine Technique

Samples of *C. elegans* grown in liquid SMM were collected after 3 days of culture, washed twice in PBS (phosphate-buffered saline) and fixed in 2.5% glutaraldehyde, in 0.1M cacodylate buffer pH 7.4, for 2 hours, post-fixed in 1% osmium tetroxide for 1 hour, followed by washing in 0.1M cacodylate buffer, pH 7.4 and dehydrated in acetone and embedded in Epon. Samples were cut in a REICHERT JUNG ultramicrotome, collected on copper grids and contrasted, observed and photographed using a JEOL CX- 100 electron microscope.

2.2.2 Ruthenium Red Cytochemistry

Cell samples of 3-day-old culture were fixed in a ruthenium red staining solution prepared according to Luft [10], (3.6% glutaraldehyde in water, 0.2 *M* cacodylate buffer, pH 7.2, and 1.5 mg/ml ruthenium red in water), post-fixed in solution of osmium tetroxide and ruthenium red. In follow samples were dehydrated in acetone and then embedded in Epon 812. Unstained thin sections were used. Samples were observed and photographed using a JEOL CX- 100 electron microscope.

2.3 Optical Fluorescence Microscopy

Cell samples collected at 3-day intervals were washed quickly with PBS and fixed for 5 min with 5% formaldehyde. They were then permeabilized with 0.5% Triton X-100 and washed in PBS. Labeling for actin was accomplished by incubation with a 1:20 dilution in PBS of FITC-conjugated phalloidin, followed by two washes with excess PBS. Labeled covers slip cultures were then placed cells-up on a glass slide, first overlaid with a drop of glycerol. Samples were observed and photographed with a Zeiss fluorescence microscope equipped with epifluorescence Optics.

3. Results and Discussion

The results obtained for the actin fluorescence study are shown in figures 1. In Figure 1A *C. elegans* hyphae of control samples exhibited fluorescence labeling at the cytoplasmic face of the cell wall, inside hyphae, and an amorphous fluorescence in cytoplasm can be observed. Globular arrangements and patches are also noted. The analysis of hyphae, submitted to 5.62 mg/L of cadmium, revealed differences in relation to control samples. Cytoskeleton preparations are characterized by the actin microfilament plates and patches that dominate the hyphae images (Figure 1B). Samples of *C. elegans* hyphae treated with 11.24 mg/L of cadmium showed

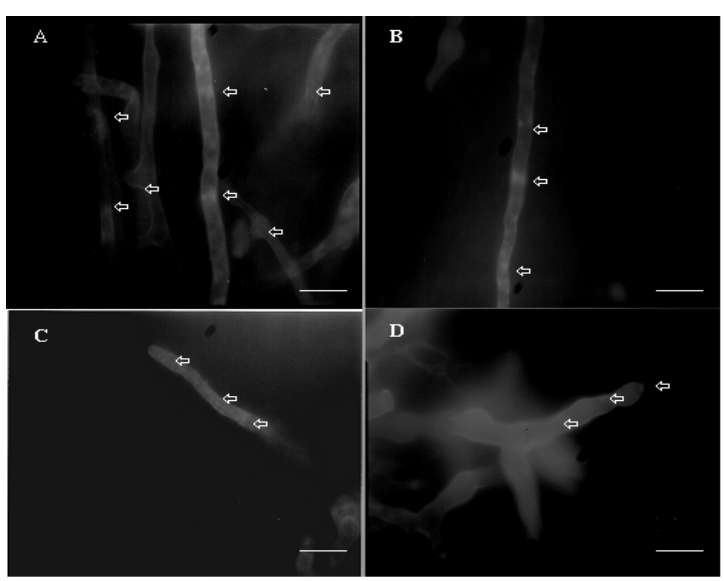

Fig. 1 Optical fluorescence of *Cunninghamella elegans* treated with FITC-faloidina. Control sample (A), Cell cultures treated with 5.62mg/L of cadmium (B), 11.24mg/L (C) and 22.10mg/L (D). Different forms of actin cytoskeleton: patches; filaments and plates distributed along the hyphae (⇦). Bar 10μm.

recognizably reduced actin fluorescence. An amorphous actin labeling along the cell wall under small aggregates and thin filaments irregularly distributed at cytoplasm are visualized (Figure 1C). On the other hand, cultures submitted to 22.10 mg/L of cadmium exhibit actin fluorescence under small and few aggregate arrangements at cell cytoplasm and some actin caps are noted (Figure 1D).

Some studies have shown that a hyphal expansion is dependent on the integrity of the actin cytoskeleton. Any alteration in the polymerization between the different actin forms could result in differences in variations of apical expansion and in normal growth [11]. Data obtained in this work revealed the highest cadmium concentrations induced the morphology of shortened, thicker and branched hyphae. These results suggest that this is the reason why the polarized growth at the apex of hyphae is reduced in treated samples compared to control. The actin forms were also modified in the presence of cadmium, indicating that actin polymerization is essential for hyphal expansion.

The ultrastructural analysis by using the routine technique at the control samples exhibit homogeneous cytoplasm, few vacuoles and electrondense inclusions (Figures 2A). *C. elegans* hyphae submitted to growth in 5.62mg/L of cadmium present, an intense vacuolization, an intense cytoplasmic granulation around vacuoles and in the membrane area. (Figures 2B). Cells submitted to growth in the presence of 11.24mg/L of cadmium exhibit a high electron-density in the cell wall and a very large number of granulations in the inside cytoplasm, vacuoles and in the cell membrane (Figures 2C). Samples submitted to 22.10mg/L of cadmium showed a granular cytoplasm as well as electron-dense bodies in the cell membrane and cell wall (Figures 2D).

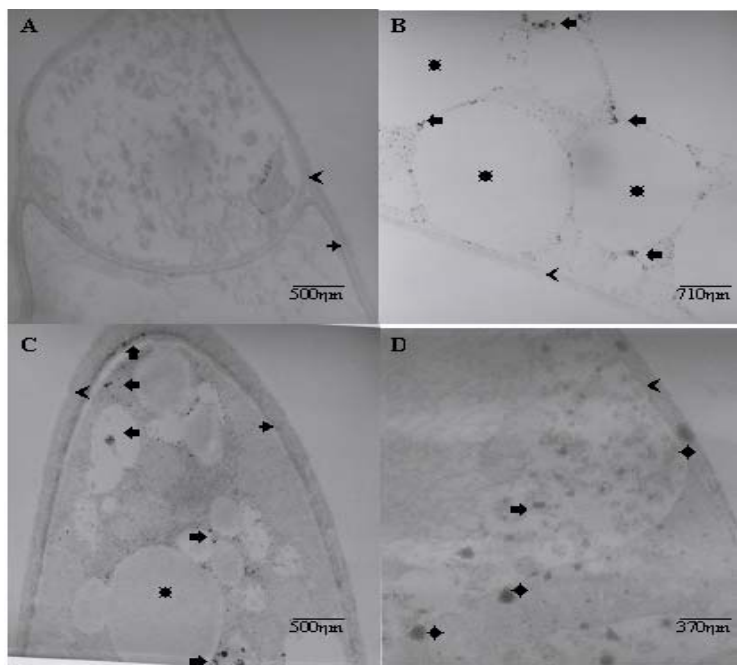

Fig. 2 Eletronmicrographs of *Cunninghamella elegans* by using the routine tecnique. Control samples (A), Cell cultures treated with 5.62mg/L of cadmium (B), 11.24mg/L (C) and 22.10mg/L (D). Cell wall (➤); Cytoplasmic membrane (➔); Vacuoles (✸); Granulations (➧); Electrondense bodies (✦).

Nishikawa et. al. [12] related the presence of cadmium deposits in the cell membrane, and in vacuoles in *Clamydomonas acidophila* and Cunningham et al. [13] revealed that *Clostridium thermoaceticum* exhibited an intense distribution of electron-dense precipitate during growth, in the presence of cadmium, and proposed this as a cellular mechanism to mediate the resistance to the metal. The intense granulation in different regions of *C. elegans* hyphae could be related to cadmium precipitation. However, it is necessary to determine the chemical composition of granules to demonstrate that *C. elegans* is able to use this mechanism as a process for cadmium tolerance.

The control samples submitted ruthenium red cytochemistry showed reaction products on the cell wall external surface and on the cytoplasmic face of cell membrane (Figure 3A). *C. elegans* hyphae submitted to growth in the presence of 5.62mg/L of cadmium exhibit an intense surface labeling and in all cell wall layers (Figures 3B). The growth of *C. elegans* in 11.24mg/L of cadmium resulted in a reduction in the cytochemical labeling of the cell surface and a increasing of reaction products in the cytoplasm and vacuoles of the cells (Figures 3C). The cells exposed to 22.10mg/L of cadmium exhibit highly electron-dense granules in the cellular cytoplasm and associated to cell membrane (Figure 3D).

The variations in the cytochemical labeling of the cell wall surface and its increase inside the cell, obtained in this study, suggesting that increasing the concentrations of cadmium induced alterations in the expression of surface polymers of *C. elegans* as well as variations in the cell permeability. Searching the literature revealed few studies about ruthenium red in fungi. Nascimento et al., [2] evaluated the Tween 80 effects on the ultrastructure of *Candida lipolytica* and revealed the surfactant-induced alterations in the distribution and concentration of surface polymers, as a response to the presence of different compound concentrations. More detailed analysis should be performed to understand cellular responses in the presence of cadmium in filamentous fungi. This paper is the first that demonstrates the effects of cadmium on the actin, surface carbohydrates and membrane permeability in *C. elegans*.

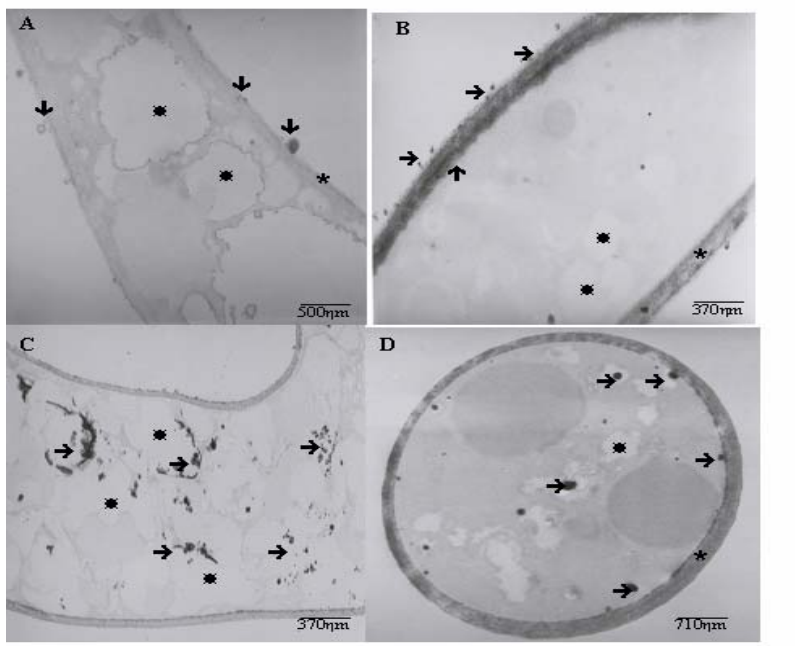

Fig. 3 Electronmicrographs of *Cunninghamella elegans* submitted to ruthenium red cytochemistry. Control culture (A), Cell cultures treated with 5.62 mg/L (B), 11.24mg/L (C) and 22.10mg/L (D). Cell wall (➘); Cytoplasmic membrane (➔); Vacuoles (✸); Reaction products (➚).

Acknowledgements The authors are grateful to CAPES, CNPq, FINEP for the financial support, and UNICAP for the use of laboratories.

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Comparative analyses of microorganisms from different high-temperature volcanic environments

J. M. González[1], and **M. C. Portillo**[1]

[1] Instituto de Recursos Naturales y Agrobiología, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

Microorganisms thriving in volcanic environments have been scarcely studied. This work presents an analysis of the microorganisms encountered in high temperature environments from two geographically distant volcanic environments, Canary Islands (Spain) and Kamchatka Peninsula (Russian Federation), with temperatures between 70 and 95ºC were sampled. Microorganisms were detected through 16S rRNA gene sequencing. Analysis based on both DNA and RNA. Interestingly, neither DNA nor RNA analysis lead to the detection of typical hyperthermophilic microorganisms. Bacterial RNA stability at high temperatures was determined and lasted just a few minutes. The presence of mesophiles in high temperature environments might be a result of a high dispersion rate. This study has serious implications on current estimates of bacterial diversity, the distribution and dispersal of microorganisms, and a high potential of microbes for colonizing novel environments.

Keywords high temperature; Canary Islands; Kamchatka; thermophiles; mesophiles; bacterial immigration; volcanic environments

1. General Remarks

Some of the most recently formed environments of our planet are those originated from volcanism representing a perfect sampling site for analyzing the colonizing potential of microorganisms. However, only microorganisms adapted to high temperature can tolerate these conditions, such as those found at hot springs and other sites related to volcanism. These microorganisms are the thermophiles, extreme thermophiles or hyperthermophiles, depending on their optimum growing temperatures [1].

Recent development of novel microbiological methods allow the detection of microorganisms without a need for culturing. Among the culture-independent techniques, molecular methods based on both DNA and RNA have been proposed for the analysis of microbial communities from natural environments. While DNA would indicate the presence of microorganisms in a given environment, the use of RNA would permit to detect those microorganisms actively playing a role in the ecosystem since the amount of RNA per cell is proportional to its metabolic activity [2]. Even if uncultured microorganisms can be cultured, the study of natural microbial communities present numerous technical problems. The huge microbial diversity detected in most environments is of critial interest [3]. At present, it is unclear which members of a microbial community are adapted to the study environment and represent stablished members of the community [4]. The methodology available today lacks the capability of detecting the characteristic microorganisms from a natural environment within a highly complex community.

The dispersal of microorganisms through our planet is a subject of great interest and has been scarcely studied [5, 6]. Due to their small size, microbes can easily propagate. Besides wind forces, aerosol formation has been reported as a major route for microbial dispersion [7] and animal transport has also been analyzed [8] although airborne microorganisms and pathogenesis transmision have been known for a long time.

Herein, an analysis focusing on microbial communities found at high temperature volcanic environments is presented. The aim is to investigate whether mesophiles (microorganisms unable to survive at high temperatures) can be found in these environments. If so, high temperature environments will contain some mesophiles and the time frame available for that detection wil depend on DNA and RNA stability, and the survival of those mesophiles under those extreme conditions.

2. Materials and Methods

Two distant volcanic areas from very distant locations were sampled. Three islands within the Canary Islands (Spain) were sampled. Samples ranged from 70 to 95ºC. These islands are located off the Western African coast and are typical examples because of their volcanic origin. Some major volcanism has occurred as closed as less than 40 years. The second sampling sites were located in Kamchatka Peninsula (Russian Federation). This Peninsula is well known by the numerous volcanes, geisers and hot springs present in that area. Some of these sites with a temperature between 75 and 90ºC were sampled.

Microorganisms were detected in base to their DNA and RNA following methods previously described [9, 10] and include the extraction of DNA and RNA, cloning and construction of 16S rRNA gene libraries, clones screening and sequencing, and data analysis. Negative controls were performed at every step during the procedure. Microbial enrichments were performed in a series of media and conditions adequate for sulfate-reducing bacteria (*Desulfovibrio* spp.), hyperthermophiles (Archaea from the O. Thermococcales) and their composition was obtained from the DSM (Microbial Culture Collection) (http://www.dsmz.de/) and other commonly reported non-selective culture media such as Nutrient Broth and Trypticase-Soy Broth. These two last media were prepared at full strength and diluted 1/10 and 1/100. These media were inoculated with samples collected at high temperatures (above 70ºC) Incubations were carried out at RNA decay was analyzed using the fluorescent dye Ribogreen and measuring the reduction of fluorescence over time at 80ºC following the methodology proposed by Gonzalez and Saiz-Jimenez [11].

3. Results and Discussion

Table 1 and 2 show the results obtained from sequencing of 16S rRNA gene fragments retrieved from the Canary Islands and Kamchatka Peninsula, respectively, from RNA extracted directly from the high temperature samples. Interestingly, most of these sequences correspond to mesophilic microorganisms, with no representatives in the thermophilic range of growth. Thus, mesophiles can be detected in high temperature environments. Among the detected microorganisms the Proteobacteria were the most frequently found bacterial group, and others like Actinobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia were significant in the studied samples. Interestingly, neither DNA nor RNA analysis lead to the detection of typical hyperthermophilic microorganisms. Enrichment cultures prepared with the same samples lead to clear bacterial growth at temperatures never higher than 50ºC corroborating the mesophilic range of growth of these microorganisms. No hyperthermophilic microorganisms where ever detected.

In order to analyze the time frame available for detecting these microorganisms using RNA, we estimated T90 (time required for a reduction of 90% of the RNA) for *Escherichia coli*, as a model bacterium, and a *Sinobacter* sp. isolated from Canary Islands, high-temperature samples. The results showed T90 lower than 5 minutes (Fig. 1). This short time frame represents a strong evidence suggesting that the arrival of microorganisms to these high temperature environments has occurred within the last few minutes. This short time also explains the survival of some mesophilic bacteria from their arrival to high-temperate sites to the time of sampling. The arrival of allochthonous microorganisms happened with at a very high rate which needs to be determine to be able to model and analyze this discovery. These results support a continuous immigration of microorganisms in natural environments.

Since mesophilic microorganisms show extremely short survival at high temperatures, hot environments might represent unique observatories to study the arrival of microorganisms. These sites are useful to analyze allochthonous microorganisms [12] lacking the ability to survive in these environments. Our experiments do not yield information on the origin of microbial immigrants. During this study, wind might have been the driven factor facilitating the dispersion of microorganisms and their arrival to high-temperature sites. The Canary Islands are exposed to frequent winds originated locally, from the Africa mainland (sandstorms are common at these islands) or even from trans-oceanic origins. Previous studies at other locations have demonstrated that microorganisms are actually dispersed through air at both local and large scales [13]. Nevertheless, the key point is that microorganisms are able to reach new environmental niches at elevated rates presenting a huge potential for new colonizations, and leading to serious implications on estimates of bacterial diversity at given environments and the spreading of genetic traits among microbial communities and ecosystems. The potential of these findings for the spreading of airborne pathogens can also be easily understood.

Table 1 Operational taxonomic units retrieved from high-temperature sampling sites from Canary Island and detected using RNA-based molecular techniques.

bp.	Taxonomic affiliation	Closest homologue	% similarity
185	Alpha-Proteobacteria, Rhizobiales, Brucellaceae	AY242688	90
392	Beta-Proteobacteria	DQ404931	99
169	Alpha-Proteobacteria, Rhizobiales, Bradyrhizobiaceae, *Bosea*	DQ986321	98
189	Bacteroidetes, Sphingobacteriales, Flexibacteraceae, *Cytophaga*	DQ188310	97
188	Bacteroidetes, Sphingobacteriales, Flexibacteraceae, *Adhaeribacter*	AJ626894	96
447	Alpha-Proteobacteria, Rhizobiales, Methylobacteriaceae, *Methylobacterium*	EF165044	98
393	Beta-Proteobacteria, Burkholderiales, Comamonadaceae, *Variovorax*	DQ432053	99
169	Alpha-Proteobacteria, Rhizobiales, Bradyrhizobiaceae, *Bosea*	DQ986321	100
168	Alpha-Proteobacteria, Rhizobiales, Bradyrhizobiaceae, *Bosea*	DQ986321	98
173	Actinobacteria	AF379034	97
391	Beta-Proteobacteria, Burkholderiales, Oxalobacteraceae	DQ064837	99
178	Actinobacteria, Actinomycetales, Corynebacteriaceae, *Corynebacterium*	AF537601	98
169	Alpha-Proteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*	DQ857187	100
174	Actinobacteria, Actinomycetales, Microbacteriaceae, *Microbacterium*	DQ922933	100
169	Alpha-Proteobacteria, Rhodobacteriales, Hyphomonadaceae, *Hyphomonas*	CP000158	97
194	Firmicutes, Bacillales, Bacillaceae, *Bacillus*	DQ904626	100
170	Epsilon-Proteobacteria, Campylobacterales, Campylobacteraceae	DQ295698	98
196	Gamma-Proteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas*	DQ904603	100
189	Bacteroidetes	AY830003	100
194	Gamma-Proteobacteria, Enterobacteriales, Enterobacteriaceae, *Escherichia*	EF418614	100
194	Gamma-Proteobacteria, Enterobacteriales, Enterobacteriaceae, *Escherichia*	EF418614	99
194	Firmicutes, Bacillales, Bacillaceae, *Bacillus*	EF377316	99
169	Alpha-Proteobacteria, Caulobacterales; Caulobacteraceae, *Caulobacter*	AF116181	100
195	Gamma-Proteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas*	EF413073	99

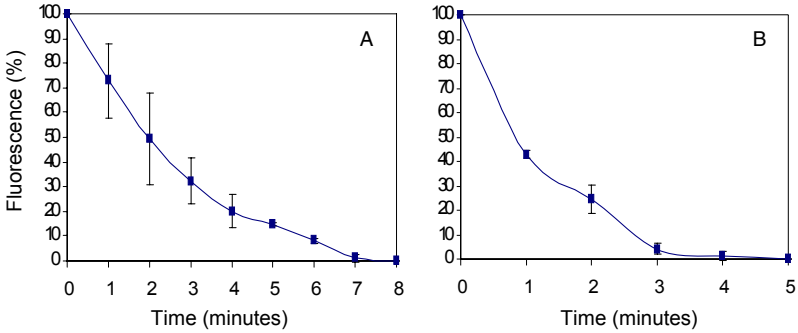

Fig. 1 RNA decay curves carried out at 80ºC for an isolate from Canary Islands hot environments, *Sinobacter* sp. (A), and *Escherichia coli* (B). Error bars indicate the SD from three replicates.

Table 2 Operational taxonomic units retrieved from high-temperature sampling sites from Kamchatka Peninsula and detected using DNA- and RNA-based molecular techniques.

bp.	Taxonomic affiliation	Closest homologue	% similarity
RNA-based analysis			
210	Beta-Proteobacteria, Burkholderiales	CP00124	100
231	Alpha-Proteobacteria, Rhizobiales, Phyllobacteriaceae, *Mesorhizobium*	BA000012	83
376	Gamma-Proteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas*	AE016784	81
124	Gamma-Proteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas*	CP000075	83
72	Beta-Proteobacteria, Burkholderiales, *Burkholderia*	CP000152	83
111	Alpha-Proteobacteria, Rhizobiales, Bradyrhizobiaceae, *Bradyrhizobium*	BA000040	84
DNA-based analysis			
740	Gamma-Proteobacteria, *Acinetobacter*		99
759	Beta-Proteobacteria	EF420213	99
726	Firmicutes, Clostridiales, *Sporolactobacillus*	DQ132980	99
726	Firmicutes, Clostridiales, *Clostridium*	DQ168835	99
344	Firmicutes, Clostridiales, *Clostridium*	DQ218319	99
234	Alpha-Proteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*	AB334774	100
375	Beta-Proteobacteria, Burkholderiales, Burkholderiaceae	EF561924	99

This study focuses on the presence of mesophilic microorganisms in high temperature environments. Previous DNA-based molecular surveys of microbial communities at volcanic environments have mentioned the dominance of Alphaproteobacteria, Gammaproteobacteria, Acidobacteria, Actinobacteria [14] or Bacteroidetes and Betaproteobacteria (Oxalobacteraceae) [15]. These bacterial groups are almost exclusively represented by mesophiles. These microbial phyla are in general agreement with the microorganisms detected during our work. Herein, a *Sinobacter* strain (Oxalobacteraceae, Betaproteobacteria) was also isolated confirming the presence of mesophiles belonging to the Oxalobacteraceae in the environments studied by [15] and in this study.

A recent computer analysis [16] has suggested that immigration can be a significant factor structuring microbial communities. This study confirms the importance of the dispersal of microorganisms in accessing new environments and provides with direct information on microbial immigration into specific spots. The presence of a significant proportion of microbial immigrants in an environment creates great difficulty to propose geographical distributions of microorganisms [17], assign microbial communities to specific environments [18, 19], and deduce the implications and functional roles of these communities on local and global processes [18]. Considering a microbial community in an environment is composed by those microorganisms well established to thrive in a habitat, allochthonous microorganisms should be subtracted from current estimates of bacterial diversity. This distinction should account for significant reductions of microbial diversity estimates at local

scales while confirming a huge global microbial diversity in our planet which is far from being determined using current experimental methods [3, 17].

Acknowledgements The authors acknowledge financing from the Junta de Andalucía to group CVI288, and greatly appreciate the collaboration of Dr. V. Soler, Dr. S. Sanchez-Moral, Dr. J. Lario, and Mr. M. Rogelio.

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Composition of halophilic bacteria survived in bioaerosol

Teruya Maki[1], Shinzi Susuki[1], Fumihisa Kobayashi[2], Makiko Kakikawa[2], Maromu Yamada[3], Tomomi Higashi[4], Chunsang Hong[5], Yutaka Tobo[1], Hiroshi Hasegawa[1], Kazumasa Ueda[1] and Yasunobu Iwasaka[2]

[1] Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan.
[2] Institute of Nature and Environmental Technology, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan.
[3] Faculty of Environmental and Symbiotic Science, Prefectural University of Kumamoto.3-1-100 Tsukide, Kumamoto 862-8502, Japan
[4] Hygiene, Kanazawa University School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan
[5] Frontier Science Organization, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan.

The Asian dust phenomena, Kosa, have possibility to carry the microbial particles (Kosa bioaerosol) influencing the microbial habitats and the human health in Japan. In this study, the bioaerosol were collected at 600 m and 2 m above ground in Kanazawa city at the Kosa coming season, April in 2007. In the culture media based on seawater and lake water, the bioaerosol at 600 m indicated the microbial growth only in the seawater medium, but no growth in the lake water medium. The bioaerosol at 2 m grew in the both media. The halophilic bacteria would maintain viable activities in the atmosphere. Furthermore, the microorganisms in the bioaerosol at 2 m indicated the better growth in this order of 0%, 3%, and 10% of NaCl concentrations, and no growth was detected in the 20% NaCl medium. According to the PCR-DGGE (denaturing gradient gel electrophoresis) analysis, same bacterial species were detected in the every NaCl concentration of culture medium, and other bacterial species could grow only in the culture media with 3% or 10% NaCl concentrations. Therefore, the halophilic or halotolerant bacteria would survive in the aerosol at 2 m above ground, and may be related to the microbial transport across the ground-atmosphere.

Keywords KOSA, Asian dust, bioaerosol, halophilic bacteria, NaCl

1. Introduction

Mineral dust aerosol particles originated mainly in the desert regions of northern China and Mongolia, influence the biogeochemical system in part of China, Korea, and Japan which are just downwind area of Asian dust sources[1]. Recent increases in the amount of Asian dust (Kosa) have the possibility that the microbial particles with the Asian dust particles (Kosa bioaerosol) are concerned with plant and animal health and cause diseases such as asthma (due to allergens) or other respiratory illness. Bioaerosol particles include virus, bacteria, fungi and pollen as well as plant and animal debris[2]. Bioaerosol research has been developing very rapidly during the last two decades[3]. Hua et al. (2007) isolated the bacterial strains from China and Japan and demonstrated that these strains were identified using the sequences analysis of 16S rDNA and house-keeping genes, gyrB and parE[4]. However, in the atmosphere of Japan, the physiological characteristics and the microbial compositions of bioaerosol have yet to be described, and the effects of Kosa on microbial habitats in Japan were unclear.

The ability of a microorganism to survive in the atmosphere is a function of its ability to withstand desiccation, extreme temperature, oxygen limitation and expose to UV radiation[5]. Halotorelant and halophilic bacteria are known to tolerate high salinity and can adapt to different stressors like high pH, extreme temperature, or desication[6]. The halobacterial population may travel across the earth for hundreds to thousands of kilometers, and several kilometers up into the atmosphere[5].

In this study, the physiological characteristics of bioaerosol collected at the Kanazawa in Japan was evaluated by physiological experiments. Next, we established the analytical method investigating the halobacterial population in bioaerosol by combining the physiological experiments and the denaturing gradient gel electrophoresis (DGGE) analysis.

2. Materials and Methods

2.1 Sampling

The bioaerosol sampling was performed in the 20th April in 2007 when the Asian dust particles were reported to be transported to Japan from China. The bioaerosol sample in the atmosphere was collected during 1 hr of day

time at 600m above the ground using a tethered balloon[7] (bioaerosol sample at 600m). The bioaerosol sample in the surface atmosphere was collected at 2 m above the ground (bioaerosol sample at 2m). For collecting the bacterial particles, we used an air pump with 0.2 μm membrane filter, and sampled 720 L of air volume.

2.2 Physiological experiments

After the bacterial particles on the filter were washed by the sterilized water, the wash solution was inoculated to the trypticase-soy-broth (TS) liquid medium which was made using seawater or lake water. The $TS10^0$ medium was composed of trypticasepeptone 17 g, phytonepeptone 5g, K_2PO_4 2.5g, and glucose 2.5g in 1 litter of water. After 14 days of incubation, the bacterial growth was determined by 550 nm of absorbance.

Furthermore, the bioaerosol sample at 2m was also inoculated to the TS meda including NaCl at the concentrations of 0%, 3%, 10% and 20%. The 2 nutrient levels of media at dilution rates of 10^0 and 10^{-1} were prepared. During the incubation, the bacterial growth was measured. The bacterial community in each culture was analyzed using the PCR-DGGE analysis using 16S rDNA.

2.3 PCR-DGGE analysis of bacterial 16S rDNA in the bioaerosol sample at 2m

On 14th day incubation of the bioaerosol sample at 2m in the TS media including NaCl at the concentrations of 0 %, 3 %, and 10 %, the bacterial particles were concentrated by centrifugation (20000 x g, 5 min) from 10 ml of samples. Bacterial DNAs were extracted and purified by phenol-chroloform extraction methods[8], and provided for the PCR amplification using the primers for PCR-DGGE analysis were F341-GC and R907[9, 10]. Thermal cycling was performed using a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan). Cycling started with 5 min at 94°C, followed by 34 cycles of 1 min at 94 °C, 1 min at 55°C and 2 min at 72°C.

The DGGE was performed with 6% acrylamide gels containing a linear gradient of denaturant from 35% to 60% (100% denaturant contained 7 mol/l of urea and 40% (v/v) formamide). Electrophoresis was carried out at 60 °C and 90 V for 16 h in 1 x TAE buffer with a DCode System (Bio-Rad, Laboratories, Hercules, CA, USA). After electrophoresis, the gels were stained cybergold, and scanned in InstaDoc system (Bio-Rad).

2.4 Sequencing of 16S rDNA and phylogenetic analysis of DGGE bands

Partial 16S rDNA sequences of PCR-DGGE bands were determined. The PCR amplicons from gel pieces of DGGE bands were purified by phenol-chloroform extraction, followed by ethanol precipitation. Nucleotides sequences were determined using a Dye DeoxyTM Terminator Cycle Sequencing Kit (ABI, CA, USA) and a DNA auto-sequencing system (ABI, Model 373A) according to the manufacture's recommended protocol. The primer 341F were used as the sequencing primers for the DGGE bands. The sequences determined were compared with a DDBJ database using FASTA SEARCH program[11]. A phylogenetic tree including all sequences was constructed according to the neighbor-joining algorithmic method[12], using the partial 16S rDNA sequences.

3. Results and Discussion

3.1 Analysis of bioaerosol comparing the bioaerosol samples at 3m and 600m

In the culture media of the bioaerosol sample at 600m, the bacteria grew in only the culture medium of seawater (Fig. 1). The bacterial growth of bioaerosol sample at 2m was confirmed in the culture medium of seawater and

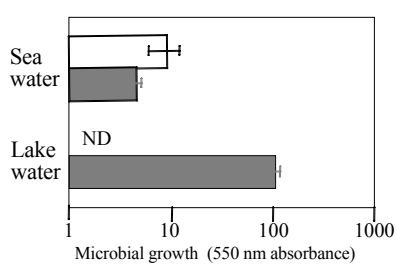

Fig. 1 Microbial growth yields in the bioaerosol samples at 600m (white bar) and at 2m (gray bar) in the TS medium of seawater or lake water. The incubation days are 14 days.

lake water. The halophilic bacteria are found to survive in extreme environments by the adaptation fro several stress such as dessication, UV irradiance, extreme temperature and oxygen limitation as well as high salinity concentration[13]. Eguchi et al. reported that same numbers of the halophilic bacteria are habiting in non-saline environments in an area surrounding Tokyo Japan, indicating the possibility that the halophilic bacteria were transported from the saline environments by Asian dust storms[14]. The halotolerant bacteria such as *Halomonas variabilis* and related bacteria are reported likely to be cosmopolitans that occur in widely various habitats[15]. Presumably, the halophilic bacterial population maintained viable activities in the atmosphere. Accordingly, the halophilic bacteria on the atmosphere at 600 m above ground of the Kanazawa city originated from the ground area, or were also transported from other areas such as China.

3.2 Physiological and phylogenetic analysis of bioaerosol in the bioaerosol sample at 600m

The bioaerosol sample at 2m was inoculated to the TS media including the NaCl at the concentrations of 0%, 3%, 10% and 20% (Fig. 2). The TS media would be more appropriate to detect the microorganisms in the

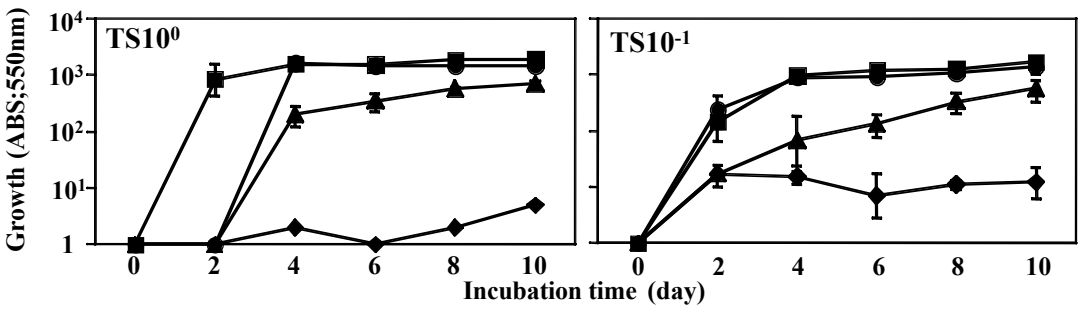

Fig. 2 Microbial growth of the bioaerosol sample at 2m in the 2 nutrient levels (dilution rate 10^0 and 10^{-1}) of culture media including NaCl at concentrations of 0% (square), 3% (triangle), 10% (circle) and 20% (diamond).

atmosphere. Some scientists used the same components of TS media for isolating the bacteria in the bioaerosol[16, 17]. The microbial growth in the TS media was lower in this order of 0%, 3%, and 10% of NaCl concentrations. The extreme halotorelant bacteria which can grow in the culture medium with 10% NaCl would occupy low amounts of biomass among the microbial community of ground area. Significant growth in the 20% culture was not detected. Presumably, the halotorelant bacteria at 3 m above the ground may be transported from atmosphere. According to the DGGE analysis of microbial community in TS media, the $TS10^0$ medium can detect more numbers of bacterial species than $TS10^{-1}$ medium (Fig. 3a), suggesting that much of bacterial population in the ground areas require high levels of nutrients. Moreover, the sequences of the bands 9 and 11 were related to the members of the genus *Microbacterium*, while those of the bands 6 and 8 belonged to the genus *Proponibacterium* (Fig. 3b). Although the bands in the similar horizontal position, they included the different bacterial species between $TS10^{-1}$ and $TS10^0$ media. Accordingly, the different bacterial species would grow between the $TS10^0$ and the $TS10^{-1}$ media. The bands 6, 8, 9, and 11 were detected in every range of NaCl concentrations of bacterial culture, while the bands 3, 4, and 5 were detected only in the culture media including 3% or 10% NaCl (Fig. 3). Both of the halophilic bacteria and the halotorelant bacteria would be able to survive in the bioaerosol of the ground area.

On the phylogenetic tree using the 16S rDNA sequences, the sequences of 5 bands detected on the DGGE gel belonged to the Gram-positive bacterial group (Fig. 4). Members of the Gram-positive bacterial group are known to form endospore to survive in the extreme environments[18]. The bacteria in the aerosol would survive in the atmosphere by forming endospores[19, 20, 21]. In fact, the some members of the Gram-positive bacterial group were identical between China and Japan, suggesting the transport of the Gram-positive bacteria[4]. The sequences of the bands 6, 9 and 11 were related to the marine bacteria such as Red Sea bacterium KT-2K12 and marine firmicute HTB096. Marine aerosol formed by the eruption of rising bubbles is known to a major source of microorganisms entering the atmosphere from seawater bodies[4, 19, 22]. Possibly, the bacterial population in the bioaerosol can grow in the seawater, and transfer across the marine environments and atmosphere.

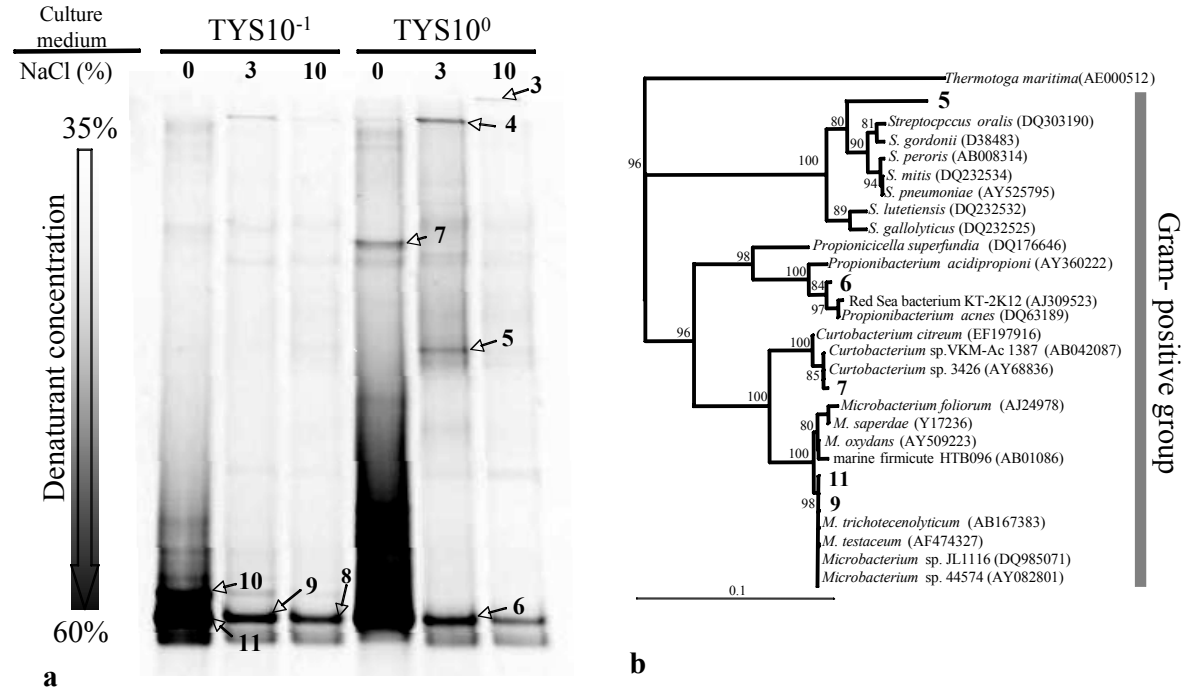

Fig. 3 a) A DGGE profile (bands patterns) of amplified 16S rDNA from the bioaerosol cultivated in $TS10^{-1}$ and 10^{0} media with NaCl at concentrations of 0 %, 3%, and 10%. b) A phylogenetic tree including partial sequences of 16S rDNA of DGGE bands. The tree was calculated from a dissimilarity matrix of ca. 400 bp alignment using a neighbor-joining algorithm. Bootstrap values larger than 50 % (after 1000 resampling) are indicated on the branches.

4. Conclusion

This study demonstrated that the halophilic bacteria survive in bioaerosol at 600m above ground, and that the halotolerant and halophilic bacteria belong to the Gram-positive bacterial group were included in the bioaerosol. Some pathogenic bacteria belong to the genus *Bucillus* were isolated from aerosol in the Kosa source region, Dunhuang City[4, 23]. The investigation for the long-distance transport of halobacterial population may elucidate the effects of Kosa aerosol on the human health and the microbial habitats in Japan.

Acknowledgements This research was supported by a Grant-in-Aid for Encouragement of Young Scientists (17710061) from the Ministry of Education, Science, Sports and Culture. The Nissan Science Foundation supports this work. This work was supported by the Global Environment Research Fund (RF-072) by the Ministry of the Environment, Japan.

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Control of *Legionella pneumophila* by disinfectants. Effects of sodium hypochlorite against persistent strains

M. T. Garcia[1], A. Martín-González[*,2] and, C. Pelaz[1]

[1] Laboratorio de Legionella, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Madrid, Spain.
[2] Departamento de Microbiología-III, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain.

[*]Corresponding author: e-mail: anamarti@bio.ucm.es Phone: +34 913944968 Fax: +34 913944964

Legionella pneumophila is the causative agent of Legionnaires' disease. *Legionella* is ubiquitous in natural freshwater ecosystems and it is frequently found in artificial aquatic systems. Due to its wide distribution and the impact on the human health, there have been applied many different physical and/or chemical treatments to control *Legionella*, especially in man-made water systems. We have reviewed the efficiency, advantages and limitations on chlorine and other disinfectants to control this important human pathogen. Besides, we have evaluated experimentally the effects of both, shock and continous treatments of chlorine (sodium hypochlorite) on viability and persistence of four different isolates of *L. pneumophila* sg1. We have demonstrated that both treatments were effective against *Legionella pneumophila* only when the bacteria were not previouly exposed to chlorine. We have also showed how under unfavourable environmental conditions such as disinfection treatments, some cells of populations might have differentiated to the viable non culturable stage. These findings should be considered in order to design effective methodologies to detect and prevent the presence of *L. pneumophila* in diverse natural and artificial aquatic habitats.

Keywords *Legionella pneumophila*, chemical disinfectants, sodium hypochorite, viable non-culturable.

1. Introduction

1.1. *Legionella* and Legionellosis

Legionella pneumophila is a gram-negative, monotrichous, non-spore-forming bacillus, belonging to the gamma subgroup of proteobacteria. It is considered as a facultative intracellular human pathogen and the causative agent of Legionnaires' disease (LD), a common form of severe pneumonia in industrialized countries that can be fatal if it is not promplty and correctly diagnosed and treated. *Legionella* is ubiquitous in natural freshwater ecosystems but there has been also detected in damp or wet soils and marine waters. Besides, it is frequently found in artificial aquatic systems that many time are the source of large LD outbreaks, such as cooling towers [1], water distribution systems (mainly hot water) of buildings [2], etc. The disease develops when susceptible individuals inhale aerosols from man-made water supply plants contaminated with the bacteria. *Legionella pneumophila* serogroup (sg) 1 is the group most frequently associated with human infections [3] and the one most frequently isolated from the environment.

1.2. Disinfection methods against *Legionella*

Due to its wide distribution, the impact on the human health and the social or economical implications such as disease victims' lawsuits or close of installations, guidelines and/or legislation have been developed and implemented to prevent and control legionellosis in many countries, like Spain [4]. Different physical and/or chemical treatments are some of the measures that have been applied to control *Legionella* in man-made water systems.

Some of these treatments kill bacteria as consequence of irreversible damages to cellular structures or functions vital for the bacteria (bactericidal effect), while others only inhibit the growth of bacteria without killing them (bacteriostatic effect). Disinfectants can be applied at high doses for shorter time (shock treatment) or at low concentrations for a long time (continuous treatment). Some times installations are treated first with primary disinfectants, that inactivate microorganisms, and then with secondary disinfectant to maintain a residual effect in the installation.

Superheating and flushing, metal ions (copper and silver), UV light, oxidizing and non-oxidizing agents are frequently used against *Legionella*. Oxidizing agents include chlorine, bromine, iodine, chlorine dioxide, chloramines, and halogenated hydantoins, ozone and hydrogen peroxide. Non-oxidizing agents include heterocyclic ketones, guanidines, halogenated amides and glycols, thiocarbamates, amines, aldehydes,

thiocyanates, organo-tin compounds and others [5]. Although the advantages and disadvantages of all these methods have been reviewed several times, no definitive recommendations have been given.

Superheating and flushing

This method has great advantages such as it does not require special equipment and the costs are minimal. However it has disadvantages such as corrosion problems, especially when it is combined with hyperchlorination. It is a time-consuming procedure and numerous personnel are involved to monitor installation. Scalding can occur. Moreover it is a temporary disinfection so re-colonization of the water system can occur.

Ultraviolet light

Ultraviolet light is a method of disinfection that has proven effective close to the point of use. The main advantages of ultraviolet light are its fast, no corrosive and no pH dependant activity, no residual sub-products, no bad smell or tastes, and an easy and cheap installation. The main disadvantages are its low activity against protozoa or biofilms, efficacy affected by higher water temperature, possible reparation of small damages and the lack of residual effect so the activity is no long-term and it is only applied as primary disinfectant in local areas where it has to be monitored. Studies about this method demonstrated its effectiveness against *Legionella* in hospitals or cooling towers, although their effectiveness is lower in some installations because of their complexity.

Metal ions

Mainly advantages are the easy installation and maintenance, an efficacy that it is not affected by higher water temperature, unlike chlorine and ultraviolet light, and that this kind of disinfection kill rather than suppressed *Legionella*, which can minimize the possibility of re-colonization. Important disadvantages of this treatment are that it is expensive and the efficacy decrease at high pH values. Moreover, *Legionella* develops a tolerance to silver ions after long exposure times. Concentrations of the metal ions have to be monitored because of their adverse impact and the electrodes must be cleaned regularly to ensure maximum performance.

Non-Oxidizing agents

Non oxidizing agents are generally more strable and longer than oxidizing biocides. Quaternary ammonium compounds (QACs) are widely used for the control of bacterial growth in clinical and industrial environments. Broad-spectrum antimicrobial activity, bactericidal activity against *Legionella,* and surfactant properties have made QACs such as benzalkonium chloride the favoured hygienic adjuncts in disinfectant cleansing formulations, and they have been increasingly deployed in domestic cleaning products over the last decade. However, some authors have demonstrated that some of these salts can cause genotoxic effects at environmentally relevant concentrations. Other oxidazing agents with bactericidal activity against *Legionella* are halogenated amides or glutaraldehyde, heterocyclic ketones or halogenated glycols, etc.

Oxidizing agents

Oxidizing agents have been widely used to disinfect potable water, cooling water, and other water systems. Chlorination (applied as sodium or calcium hypochlorite or chlorine dioxide) and ozonitation are two methods effective against *Legionella.* However, they are not favoured because of high expense, marginal efficacy, corrosion of piping, release of carcinogenic products into the drinking water and difficulties to maintain a residual disinfectant level because their concentration decreases at high temperatures or pH values and, in the case of chlorine, also in presence of organic products. Popularity of chloramines, especially monochloramine, has increased as an alternative to chlorine because of recent concerns for the production of harmful chlorination by-products. Moreover, they enter better into biofilm than free chlorine, resulting in better inactivation of bacteria in the biofilm. However, they must be produced in situ and their efficacy decrease at high pH values. In addition, the effectiveness of organic chloramines could be slower against *L. pneumophila* than free chlorine. Bromine and iodine has also been used in disinfection treatments. Bromine has been used in swimming pools and cooling water, but it has not been recommended for potable water, and iodine has been used to disinfect potable water and swimming pools. However, bromine seems to be less effective than chlorine against *L. pneumophila* and there is little information available on the use of iodine for control of *Legionella*. Other products used against *Legionella* are hydrogen peroxide and potassium permanganate. However they are weaker disinfectants than chlorine and ozone and they have not been widely accepted and used for disinfection. The search on new disinfection methods continues nowadays. Recently, a new ultrasonic treatment system using a TiO2 photocatalyst was used against *Legionella pneumophila*, improving the disinfection process [6].

1.3. Persistence of *Legionella* after treatments

Persistence of epidemic *Legionella* strains has been demonstrated into water systems of buildings LD associated for years, even though they were disinfected over the time [7]. Therefore, when an installation is colonized by *Legionella*, total eradication of bacteria is almost impossible despite treatments. Several factors could explain this lack of efectiveness. These methods are not always efficient or easy to establish [8]. Facility's intricate piping system can hinder the effectiveness of thermal or chemical disinfection treatments [9]. Protozoa, the

natural reservoirs of *Legionella*, and biofilms, where bacteria is quite frequent, protect bacteria against disinfectant activity and support its growth in installations [10]. Short-term effect of some disinfectants makes possible the re-colonization. There is a lack of available information on many disinfectants regarding to effective doses and exposure times, action mechanisms, secondary effects, resistances, activity in biofilms, etc. Available tests for screening the activity of disinfectants are not standardized, despite it has been shown that factors such as culture conditions, or presence of organic matter can modify the activity of some disinfectants [11]. Bacteria exposed to bacteriostatic disinfectants or to an insufficient concentration of bactericidal products can remain alive and retain the potential to become resistant or promulgate any resistance selected by the exposure to the disinfectant. Like others many gram-negative and some gram-positive bacteria, under stress or unfavourable environmental conditions, vegetative cells can differentitate to the viable non-culturable stage (VNC) that are not detected with routine tests.

2. Experimental effect of sodium hypochlorite against persistent *Legionella* strains

Chlorine is the best known disinfectant. It has been widely used to disinfect water systems, cooling towers, and other water systems. It is applied in shock or continuous treatments, following the recommendations of different guides. However, it has been showed a lack of effectiveness of hyperchlorination in a definitive elimination of *Legionella* from installations [12]. We have evaluated experimentally the effects of both shock and continuus chlorine treatments (sodium hypochlorite) on viability and persistence of four different *L. pneumophila* sg 1 strains.

2.1 Material and methods

Bacteria

We have used four different *L. pneumophila* sg1 strains: one clinical isolate and three isolates that were previously exposed to chlorine: the previous mentioned clinical isolate but 24h treated with 16 ppm sodium hypochlorite in our laboratory and two environmental isolates recovered from a cooling tower where several hyperchlorination treatments were applied during several months.

Minimal Inhibitory Concentration (MIC)

This was determined by a conventional macrodilution method in BYE broth [13]. Cells growth on BCYE-α agar for 3 days at 37^{o}C were suspended at (1-3) x 10^8 cfu/mL (0.2 O:D; $\lambda_{420\ nm}$) in buffered yeast extract (BYE) broth containing a series of two-fold dilutions of sodium hypochlorite (2-2048 ppm). The MIC value (expressed as ppm) was the lowest concentration of disinfectant without visible growth after 48 hours of incubation at 30^{o}C. Negative controls were included following the same method but without disinfectant. Each assay was repeated twice.

Minimal Bactericidal Concentration (MBC)

This was calculated following the described method [14]. Briefly, aliquots of treated cultures with no visible growth in the MIC assays were plated onto BCYE-α and incubated for 48 h at 35^{o}C for total viable counts (TVC). MBC values, expressed as ppm, corresponded to the lowest concentration of disinfectant where viable counts were lower than 0.1% of untreated samples. Each assay was repeated twice.

Survival assays

Bacteria grown on BCYE agar for 48 h at 35^{o}C were suspended at (1-3) x 10^8 ufc/mL in 1L of hard water and treated with sodium hypochlorite at dose and exposition time recommended by manufacturers in continuous and shock treatments of installations. Therefore, some suspensions were treated with 2 ppm of Sodium Hypochlorite for 2 months, measuring and adjusting the biocide concentrations each day as needed (continuous treatments). Other suspensions were exposed to 20 ppm of Sodium Hypochlorite for 3 hours, removing then the disinfectant by washing (shock treatments). Suspensions were incubated for 2 months at 35 ^{o}C. Control assays, without disinfectant, were also included. The number of bacteria was followed for 2 months by colony enumeration on MWY agar plates. To detect viable non-culturable *Legionella* in suspensions that looks sterilized by the treatments described above, 10 mL aliquots of the mentioned suspensions were centrifugated at 4000 rpm for 20 min. After removing the supernatant, pellet was suspended with BYE and incubated for 14 days at 30^{o}C. Number of bacteria was determined as Log (cfu/mL) at 0h, 1d, 2d, 4d, 7d and 14 days by colony enumeration on MWY agar plates.

2.2 Results and discussion

Table 1 shows how all isolates have the same MBC (Minimal Bactericidal Concentration), 1024 ppm of sodium hypochlorite but the MBC (Minimal Inhibitory Concentration) was slightly higher in environmental isolates, 1024 ppm, than in the non previously exposed to disinfectant clinical isolate, 512 ppm (Table 1).

Table 1. Susceptibility of *L. pneumophila* sg 1.

Strain	MIC	MBC
Clinical	512 ppm	1024 ppm
Environmental (September)	1024 ppm	1024 ppm
Environmental (December)	1024 ppm	1024 ppm

Clinical isolate treated with chlorine disappeared within 2 months because Legionella was not detected in hard water or BYE aliquots. Pretreated clinical isolate not survived when continuous chlorine treatment was applied but it persisted as viable non-culturable after 2 months of shock treatment (Figure 1).

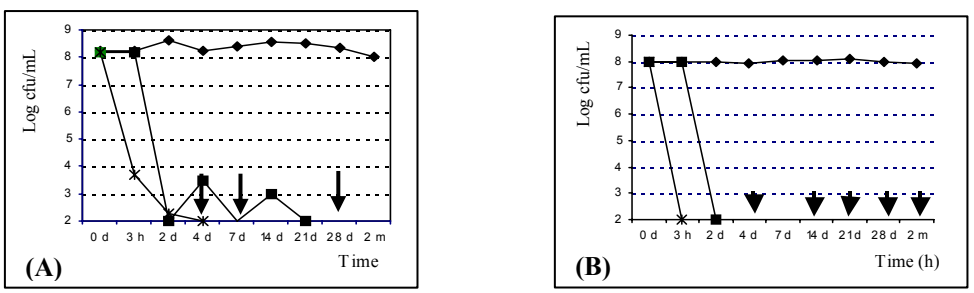

Figure 1. *Survival of L. pneumophila sg* 1 strains *after laboratory chlorination.* (♦) without treatment, (■) during continous treatment with 2 ppm of sodium hypochlorite, (+) after 3 hours of shock treatment with 20 ppm of sodium hypochlorite, (↓) Growth detected with aliquot at this time point in BYE. (A) No pretreated with chlorine. (B) Pretreated with chlorine

Environmental isolates recovered from hyperchlorinated installation persisted after 2 month of continuous or shock treatment (Figure 2).

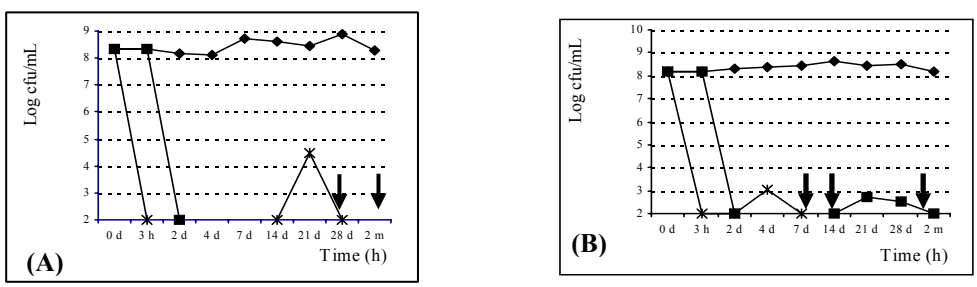

Figure 2. *Survival of L. pneumophila sg 1 strains after environmental chlorination.* (♦) without treatment, (■) during continous treatment with 2 ppm of sodium hypochlorite, (+) after 3 hours of shock treatment with 20 ppm of sodium hypochlorite, (↓) Growth detected with aliquot at this time point in BYE. (A) No pretreated with chlorine. (B) Pretreated with chlorine

Both chlorine treatments (shock or continuous) eradicated *Legionella pneumophila* after 2 months only when the bacteria were not previously exposed to chlorine. Under these unfavourable environmental conditions, some cells of populations might have differentiated to the viable non cultured stage. These data agree with previously reported in other species of gram-negative human pathogenic bacteria, such as *Helicobacter pylori* and *Arcobacter butzleri* exposed to free chlorine [15, 16]. In the VNC stage, the bacteria are more resistant to disinfectants and other antimicrobial agents but they may be more virulent and retain capacity to cause infections. Consequently, the pathogens could survive to disinfection practices normally used in drinking water treatment and microbial prevention in artificial installations and they were not also detected by usual culture

methods. It is specially relevant in the case of *Legionella pneumophila* because in previous studies it has been demonstrated that *Acanthamoeba polyphaga*, and probably other free-living protozoa, can resuscitate viable non cultured forms of this bacteria after disinfection with sodium hypochlorite [17].

3. Future challenges

Legionellosis is generally considered a preventable illness as controlling or eliminating the bacterium in installations will prevent cases breaking out. However, current treatments fail many times to eradicate the bacteria. In order to avoid the persistence of epidemic strains in installations is fundamental to design standard susceptibility tests useful not only to predict the effectiveness of disinfectants applied but also to detect possible resistances in persistent strains. We must also find effective methods to detect and prevent the presence of the bacteria not only in the viable and cultured stage but also in the viable and non cultured phase.

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Degradation of phenols by *Fusarium moniliforme*

I. Stoilova[1], A. Krastanov[1,*], H. Yemendzhiev[2], Z. Alexieva[2]

[1] Department of Biotechnology, University of Food Technologies, 26, Maritza Blvd., Plovdiv 4002, Bulgaria
[2] The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bontchev Str., bl. 26, Sofia 1113, Bulgaria

[*]Corresponding author: e-mail: abt-kr@rocketmail.com, Phone: +359 32 603607

The ability of the fungus *Fusarium moniliforme* to degrade phenol, catechol, 2,4-dichlorophenol and their mixtures was investigated in the present study. The biodegradation studies were performed in a liquid medium with the phenolic compounds as a sole carbon and energy source. It was found that temperature of 25 oC was optimal for 100% degradation of phenol, 2,4-dichlorophenol and catechol in concentration of 1.0 g/L. In case of mixtures of phenols in concentration of 1.0 g/L phenol + catechol was degraded 100 %, phenol + 2,4-dichlorophenol - 55% and 2,4- dichlorophenol + catechol only 35 %. Our study shows that investigated phenols were metabolized by the β-ketoadipate pathway, through ortho-fission of catechol.

Keywords Fusarium moniliforme, phenol degradation, phenol, catechol, 2,4-dichlorphenol

1. Introduction

Phenol and its derivatives are widespread pollutants of the wastewater from many industries, like pharmaceutical, oil processing, coal refining, etc. Phenol is a toxic and dangerous substance even in low concentrations [1, 2, 3] and effective processing methods are necessary for the reduction of phenol concentration in wastewater.

Form all processing methods the biological ones are especially attractive, because they have the potential to degrade phenol and its derivatives completely.

The microbial metabolism of the aromatic compounds is studied in detail, but most of the information for the metabolic pathways for aromatic degradation is based on investigations on bacteria. The degradation of aromatic compound in bacteria follows the pathways of well known catalytic reactions. These compounds are transformed into one of the hydroxyaromatic compounds, like catechol, 2,5-dihydroxybenzoic acid and protocateholic acid. These mediators undergo further *orto*- or/and *meta*- ring cleavage of the ring, after which are formed metabolites for the Crebs cycle, like pyruvate, acetate, succinate and acetyl-CoA [4].

The investigations with filamentous fungi demonstrate their important role in the aromatic compound recycling in the biosphere, including phenol. Molds like *Fusarium flocciferum* [5, 6, 7], *Aspergillus fumigatus* ATCC 28282 [8], *Aspergillus fumigatus* [9], *Graphium sp*. FIB4 [10] are studied for their potential phenol-degrading activity. In all cited studies phenol was degraded along the β-ketoadipate pathway through *orto*-cleavage of catechol.

Like all other metabolic pathways, he biodegradation of phenol can be influenced by different factors, for instance: substrate concentration, which in high concentrations can be toxic for the species capable of using it as a substrate; presence of additional carbon source; process temperature, microbial quantity and quality; pH of the medium, etc.

In the present study we investigated the biodegradation characteristics of *Fusarium moniliforme* strain regarding different phenolic compounds and its mixtures. The experiments were performed at two different temperatures of cultivation.

2. Materials and methods

2.1. Microorganism

A strain of *Fusarium moniliforme* from the collection of the Department of Biotechnology, University of Food technologies, Plovdiv – Bulgaria was used throughout this study. The organism was grown on slants of a malt extract medium. The organism was allowed to grow on the slants for 7 d at temperature (28°C - 30°C) and then stored at 4±1°C for further use.

2.2. Biodegradation media

Biodegradation was conducted on Chapek-Dox salts medium containing, as follows (in %): $NaNO_3$ – 0.2, KH_2PO_4 – 0.1, KCI – 0.05, $MgSO_4.7H_2O$ – 0.05, $FeSO_4.7H_2O$ – 0.001. Three phenolic compounds were used individually or mixed as single carbon and energy sources: phenol, 2,4-dichlorophenol, catechol, phenol + 2,4-dichlorophenol, phenol + catechol, 2,4-dichlorophenol + catechol. The total concentration of phenolic compounds in each sample was 1 g/l. In the mixed carbon sources the concentration was shared equally (0.5 g/l phenol and 0.5 g/l contributed by the second phenolic derivate). The starting pH of culture media was 4.5.

2.3. Growth of the microorganism

A spore suspension was prepared from a 15 d *Fusarium moniliforme* culture, which contained 4.10^7 spores/ ml medium. 25 ml of the medium, containing 1g/L catechol + the Chapek-Dox salts was poured in 260 ml flasks. After 3 d of cultivation the produced biomass was centrifuged under sterile conditions at 5000 rpm for 20 min and was washed two times with sterile deionized water. This biomass was used as an inoculum of the biodegradation media. The biodegradation was conducted at temperatures of 25°C and 30°C.

To follow up the growth phases of the organism, flasks containing 25 ml liquid medium were agitated on a shaker (240 rpm) at a temperature of 30°C and 25°C. Samples were taken at every 24-hour interval and centrifuged at 5000 rpm for 20 min to separate cells. The dry weight of the cells was determined by an ULTRA X apparatus for drying.

2.4. Analytical methods

The concentration of phenolic substrates was determined using Folin-Ciocalteu reagent, with gallic acid (0.01%) as a standard. 0.5 ml of the Folin-Ciocalteu reagent and 2.0 ml 10% NaCO3 (w/v) were added to 1.0 ml of the test solution. After 10 min, 1.0 ml of the resulting solution was diluted to 5.0 ml with water, and the absorbance was read at 620 nm [8]. Residual phenol in the samples was determined after centrifugation of the cultural medium for 20 min at 5000 rpm.

2.5. Enzyme analyses

Phenol hydroxylase – by spectrometry at 340 nm, measuring the oxidation of NADPH in the presence of phenol [11].

Catechol-2,3-dioxygenase – by spectrometry at 375 nm, quantifying the amount of 2-hydroximuconic semi-aldehyde formed [12].

Catechol-1,2-oxygenase – by spectrometry at 260 nm, measuring the levels of the emerging *cis, cis* – muconic acid [13].

3. Results and discussion

Earlier was established that the investigated *Fusarium moniliforme* strain degrades and utilizes catechol easier than any other phenol derivate. With the purpose to obtain enough adapted biomass for inoculation of the media we cultivated the cells in a medium complemented with 1g/l catechol.

For the biodegradation of the phenolic compounds and mixtures at 30°C was used initial biomass concentration of 0.160 g/l. the catabolism of phenol, 2,4-dichlorphenol and catechol is depicted on Fig. 1. The figure shows that the catechol was completely removed for 2 days, 88% of the 2,4-chlorphenol was degraded for 3 days and 28% of the phenol for 3 days. There was a correlation between the degree of biodegradation of the phenolic compound and the increase of biomass, which demonstrates the capability of the strain to not only degrade, but also assimilate the investigated compounds. The increase of biomass was greatest in a medium containing catechol – it reached 0.920 g/L (an increase of 0.760 g/L). The growth was weaker in a medium containing 2,4-dichlorphenol – an increase of 0.190 g/L, and the weakest in a medium with phenol – 0.090 g/L.

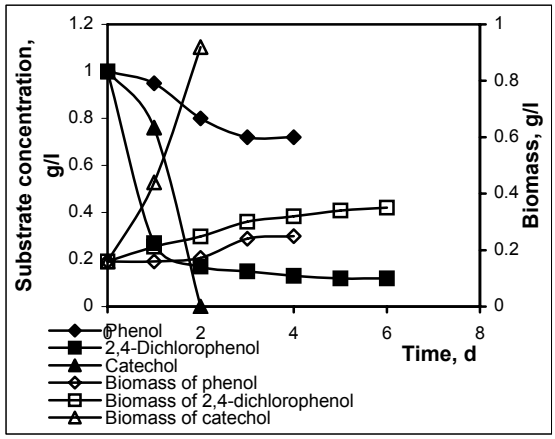

Fig. 1. Biodegradation of phenolic compounds with Fusarium moniliforme biomass at 30°C

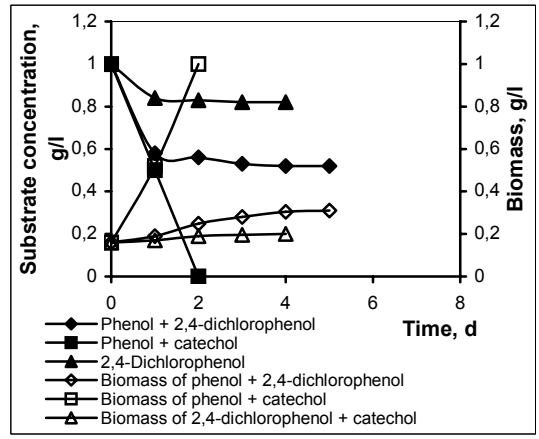

Fig. 2. Biodegradation of mixtures of phenolic compounds with Fusarium moniliforme biomass at 30°C

The catabolism of phenol mixtures with *Fusarium moniliforme* was studied at 30°C (Fig. 2). For 2 days was achieved 100% degradation of the mixture phenol+catechol. For 4 days were removed 48% of the phenol+2,4-dichlorphenol mixture and weakest biodegradation was observed in the 2,4-dichlorphenol+catechol mixture – 18% for 3 days. These significant differences in the biodegradation of the individual phenolic compounds and their mixtures are probably due to the emergence of co-metabolism or an intense inhibitory effect on the culture when the toxic substrates were mixed. For example, phenol and catechol in a mixture increase the process of degradation compared to the individual degradation of the compounds. On the contrary, 2,4-dichlorphenol and catechol, which are completely catabolized by *Fusarium moniliforme* in concentrations of 1 g/l, in a combination were degraded less than 18%. In the case of the last two mixtures – phenol+2,4-dichlorphenol and 2,4-dichlorphenol+catechol, the process of biodegradation stopped after a certain level of degradation was reached. There is a correlation between the degree of catabolization of the phenol mixture and the accumulated biomass. The most significant accumulation was determined in a medium with phenol+catechol – an increase of 0.840 g/L and the smallest increase was detected in a medium with 2,4dichlorphenol+catechol – 0.040 g/L.

The optimal conditions for degradation of the different aromatic compounds with the investigated *Fusarium moniliforme* strain were not determined until the present moment. The data demonstrated until this point are results from experiments conducted at the optimal temperature for development of the strain in an adequate nutrition medium. We assumed it is possible, that the low degree of biodegradation of phenol and the mixture 2,4-dichlorphenol+catechol is a result from a distinction between the optimal temperature for cell growth and for degradation of toxic aromatic compounds.

The next set of experiments was carried out at a 5 degree lower temperature - 25°C.

The biodegradation characteristics of *Fusarium moniliforme* for the three investigated phenolic compounds at 25°C are presented on Fig. 3. We achieved 100% degradation of phenol and 2,4-dichlorphenol for 5 days and 100% degradation of catechol for 2 days. The rate of phenol biodegradation differed form that of the other two compounds. Until 3 d were degraded only 18% of the initial phenol concentration and during the next 2 d were metabolized the other 82%. In the case of catechol the removal was realized with greatest rate between 2 d and 3 d, in 2,4-dichlorphenol during 1 d were degraded 72% of the initial concentration and after that the degradation continued slowly until 5 d.

The biomass growth correlates with the enhanced metabolism of the phenolic compounds. The biggest quantity of biomass was accumulated in the catechol containing medium and from an initial concentration of 0.140 g/L reached 0.900 g/L or an increase of 0.760 g/L. the biomass growth on the phenol containing medium was 0.210 g/L and in the 2,4-dichlorphenol medium – 0.160 g/L.

These results confirmed the presumption that the optimal temperature for phenolic compounds degradation differs from the optimal temperature for the strain cultivation in a glycogen substrate medium. It was established that a temperature of 25 °C is more favorable for the biodegradation of the investigated aromatics. Catechol degradation was not influenced by the temperature change most likely because of the previous strain adaptation to catechol in the inoculum.

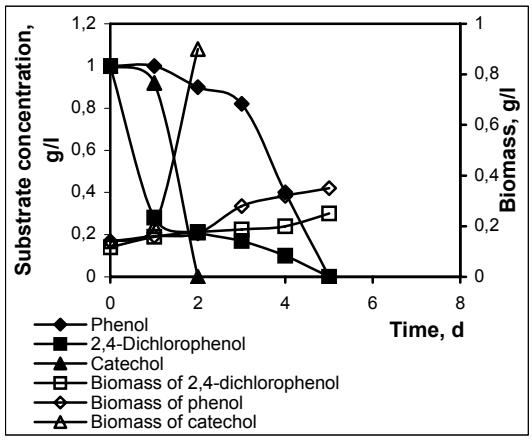

Fig. 3. Biodegradation of phenolic compounds with Fusarium moniliforme biomass at 25°C

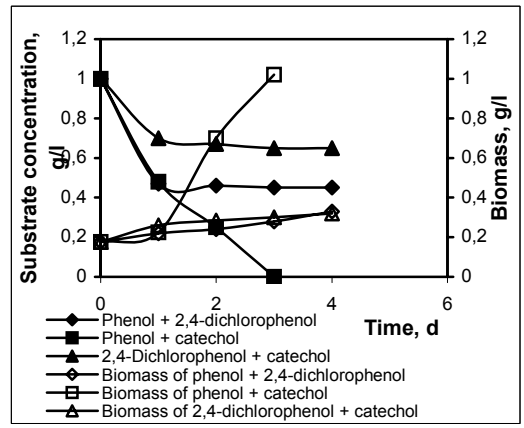

Fig. 4. Biodegradation of mixtures of phenolic compounds with Fusarium moniliforme biomass at 25°C

The catabolism of phenol mixtures with *Fusarium moniliforme* biomass at temperature 25 °C is depicted on fig. 4. the investigated strain metabolized 100% of the mixture catechol+phenol for 3 d, 55% of the mixture phenol+2,4-dichlophenol for 3 d and 35% of 2,4-dichlophenol+catechol for the same period of time. The lower strain cultivation temperature – 25 °C, influenced in a greater extend the biodegradation of 2,4-dichlophenol+catechol. The degradation rate of this mixture was almost twice as high as that at 30 °C. The biodegradation of phenol+2,4-dichlophenol also increased – it was 55% compared to the 48% at 30 °C. phenol+catechol were metabolized completely at both temperatures, but at 25 °C the time needed to achieve this level of degradation increased from 2d to 3 d. It can be noted that while all 3 phenolic compounds were catabolized completely by *Fusarium moniliforme*, the degradation and assimilation of the corresponding mixtures with the same strain was hampered although their concentration was 0.5 g/L each.

These investigations also revealed the distinct correlation between the biodegradation degree and the biomass accumulation. In a phenol+catechol medium the biomass increased significantly – from 0.176 g/L to 1.02 g/L (an 0.844 increase). The accumulation of biomass was lower during the catabolism of the other two mixtures – 0.154 g/L for the phenol+2,4-dichlophenol mixture and 0.144 g/L for the 2,4-dichlophenol+catechol mixture. The values for biomass at 25 °C and 30 °C show a significant difference in the case of 2,4-dichlophenol+catechol. At 25 °C the strain accumulates 3.6 times more biomass, which corresponds to 2 times better metabolization of the phenolic mixture in comparison with the higher temperature. In table 1 are presented the data, which characterize the process of degradation and assimilation of the investigated phenol derivatives and their mixtures at different cultivation temperatures. From the comparison of the obtained results can be concluded, that the reduction of temperature has a beneficial effect on the degradation of these compounds and that his phenomenon is more obvious in the case of the mixtures.

Table 1. Influence of temperature on the process of biodegradation with Fusarium moniliforme

Compounds	Degradation, %		Biomass accumulation, g/l		Time, days	
	25°C	30°C	25°C	25°C	30°C	25°C
Phenol	100	28	0.210	0.090	5	3
2,4 chlorophenol	100	88	0.160	0.190	5	3
Catechol	100	100	0.760	0.760	2	2
Phenol+catechol	100	100	0.840	0.840	3	2
Phenol+2,4-chlorophenol	55	48	0.154	**?**	3	4
2,4 chlorophenol+catechol	35	18	0.144	0.040	3	3

The study of the enzymes degrading phenol and its derivatives showed, that in *Fusarium moniliforme* the biodegradation is performed by the enzymes phenol hydroxylase and catechol 1,2-oxygenase (table 1). The analyses revealed an absence of the enzyme catechol 2,3-oxygenase. This means that the biodegradation is completed by the β-ketoadipate pathway.

Acknowledgements: The support by The National Science Fund, Bulgaria is gratefully acknowledged (project N VU-B-207/2006).

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Degradative potential of marine bacterial isolates from the aquatic plant *Posidonia oceanica*

Ester Marco-Noales[*,1], Mónica Ordax[1], Neus Garcias-Bonet[2], María M. López[1], Núria Marbá[2], and Carlos M. Duarte[2]

[1] Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, Carretera Moncada – Náquera, km 4.5, 46113, Moncada, Valencia, Spain.
[2] IMEDEA (Instituto Mediterráneo de Estudios Avanzados), Miquel Marquès 21, 07190 Esporles, Islas Baleares, Spain

[*]Corresponding author: e-mail: ester.marco@ivia.es, Phone: +34 963424000

The marine angiosperm *Posidonia oceanica* develops extensive meadows across the Mediterranean coast, with an important ecological role in the sea environment. These meadows are declining, probably due to a complex combination of both physical and chemical factors. However, biotic factors could also be contributing in some extent to seagrass decline. Enzymatic activities of bacterial isolates belonging to several genera recovered from different tissues of *P. oceanica* were analyzed, both by the miniaturized system API ZYM, and by hydrolysis of plant components in agar plates. The results indicate that isolates of *Pseudoalteromonas* spp. and *Alteromonas* spp. have the greatest enzymatic activity. A high degradative potential suggests that some bacteria recovered from *P. oceanica* could cause lesions in the plant, making it more susceptible to the attack by pathogenic bacteria or other microorganisms that can use these portals of entry, or increasing its susceptibility to any other stress factor.

Keywords enzymatic activity; starch; pectin; cellulose; Pseudoalteromonas spp.; Alteromonas spp.

1. Introduction

Posidonia oceanica (L.) Delile is the dominant seagrass in the Mediterranean Sea [1], representing one of the most productive ecosystems [2]. It develops extensive, highly productive and millenary submarine meadows across the Mediterranean coast [3, 2], playing an important ecological role in the marine environment through the high primary production, the varied fauna and microbiota living in, and as a barrier for preventing coastal erosion. *P. oceanica* meadows are declining throughout the Mediterranean as a consequence of not fully known factors, consequence of human activities and natural events [4, 5]. In fact, although biotic factors could also have some influence, the potential contribution of bacteria to *P. oceanica* meadows decline or health has not been addressed.

Some bacterial microbiota associated with the aquatic plant *P. oceanica*, in different meadows of Western Mediterranean Sea, has been recently identified [6]. The most abundant isolates recovered by culture from rhizome, roots and leaves of this seagrass, belonged to *Vibrio*, *Pseudoalteromonas*, *Alteromonas* and *Marinomonas*, genera reported in diverse marine habitats. The involvement of some of these genera, such as *Alteromonas* and *Pseudoalteromonas*, in pathogenicity for fish and algae has been demonstrated [7, 8]. However, little attention has been given to bacterial species that might affect the viability of marine angiosperms, despite increasing studies on bacterial activity and production in seagrass meadows [9, 10].

Alteromonas and *Pseudoalteromonas* are two close genera resulted from the division of the genus *Alteromonas* [11]. *Pseudoalteromonas* spp. are frequently found in association with the surfaces of eukaryotic hosts, and are also known to produce a variety of extracellular compounds [12]. In terrestrial environments, the secretion of a wide range of enzymes able to degrade components of the cell wall of vascular plants and other cellular components has a significant role in bacterial diseases of plants. These enzymes are divided in three groups: pectolytic enzymes, cellulases and proteases. The possession of such enzymes allows the entry of the bacterium in the plant. Scarce information exists on these aspects in aquatic plants.

In this study, we have evaluated the enzymatic potential of a wide collection of bacterial isolates belonging to the most abundant genera found in association with diverse *P. oceanica* meadows located in Balearic Islands (Spain). The enzymatic abilities exhibited by some of these isolates suggest that some bacterial species can be causing some kind of damage to the plant, making it more susceptible to any stress factor.

2. Materials and Methods

2.1. Sample collection

Five meadows located in Balearic islands (Western Mediterranean) were sampled in 2004: Illetes, Porto Colom, Magalluf, Cala d'Or and Pollença. The location is shown in Fig.1. Leaves, roots and internal part of rhizome were comminuted in small pieces in Artificial Sea Water (ASW), and then the extracts incubated approximately 2 h with shaking at 25ºC. Strains were routinely cultivated on Marine Agar (MA, Becton-Dickinson) at 25ºC.

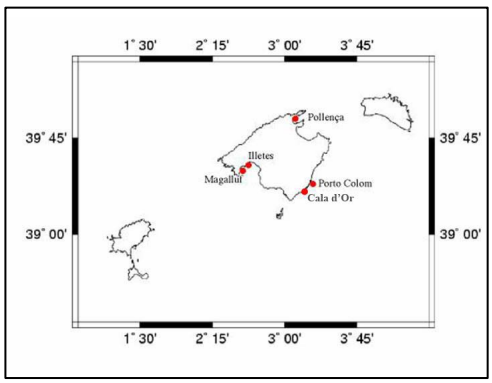

Fig. 1. Map of Mallorca island (Baleares, Spain), showing the location of the five *P. oceanica* meadows sampled in 2004, where the bacterial isolates used in this study were recovered.

2.2. Bacterial strains

Isolates from samplings were identified by sequencing of ribosomal 16S DNA (rDNA). Amplification of the rDNA 16S was performed as described by Martínez-Murcia *et al.* [13]. The DNA sequence was determined by direct sequencing of the PCR product on an ABI 3100 Avant sequencer (Applied Biosystems). A selection of isolated bacterial strains belonging to several genera and/or species were assayed in the present study for evaluating their enzymatic activity by different approachs (Table 1).

Table 1 Taxonomic classification of the bacterial isolates used in this study.

Number of isolates assayed	Bacterial species or genera
45	*Pseudoalteromonas* sp. / *P. atlantica* / *P. carrageenovora* / *P. agarivorans* / *P. aurantia* / *P. porphyrae*
32	*Alteromonas* sp. / *A. atlantica* / *A. carrageenovora* / *A. stellaepolaris* / *A. macleodii*
1	*Glaciecola mesophila*
1	*Agarivorans albus*
9	*Shewanella* sp. / *S. pacifica* / *S. putrefaciens* / *S. frigidimarina*
33	*Marinomonas* sp. / *M. mediterranea* / *M. communis* / *M. vaga*
6	*Marine bacterium TW-9*
53	*Vibrio* sp. / *V. splendidus* / *V. pomeyori* / *V. aesturianus* / *V. nigripulchritudo* / *V. gazogenes* / *V. tasmaniensis* / *V. pelagius* / *V. natriegens* / *V. parahaemolyticus* / *V. alginolyticus* / *V. tasmaniensis* / *V. cyclitrophicus*
3	*Neptunomonas* sp.
2	*Sulfitobacter pontiacus*

2.3. Enzymatic assays

2.3.1. Qualitative methods.

The amylase, pectinase and cellulase activities were assayed on MA plates supplemented with soluble starch (Sigma), citric pectin (Sigma) or carboxymethyl cellulose (Sigma) at 1% final concentration. Activity of isolates

was revealed, after 48 h incubation at 25ºC, by the appearance of a white (pectinase) or clearance (cellulose, amylase) halo surrounding the colonies, directly or after addition of Lugol's solution in the case of starch.

2.3.2. Semi-quantitative methods.

Selected isolates were also evaluated by the miniaturized system API ZYM (BioMèrieux), that allows the semi-quantitative detection of the following enzymes: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase y α-fucosidase.

3. Results

3.1. Bacterial species present in *P. oceanica* meadows

In the sampling of spring season, most of the recovered isolates belonged to *Vibrio* spp., *Marinomonas* spp. and *Pseudoalteromonas* spp. Thus, in three of the four sampled meadows, 10-60% of total isolates were of *Vibrio* spp. and *Pseudoalteromonas* spp., and 8-30% of *Marinomonas* spp. In autumn, *Alteromonas* spp. were the most represented ones, with 20-45% of the total isolates, followed by *Vibrio* spp., isolates with high homology to a group of marine bacteria (Tw-9) not identified yet in the data base, and *Pseudoalteromonas* spp. Other genera, such as *Shewanella*, *Marinobacter*, *Neptunomonas*, *Sulfitobacter* and *Halomonas* were also represented but with low numbers of isolates.

3.2. Degradation of plant components in agar plates

Almost 95% of isolates from *Pseudoalteromonas* spp. were able to degrade starch, around 55-65% of those belonging to *Alteromonas* spp. and *Vibrio* spp., and only some strain from *Marinomonas* spp. (Fig. 2). The ability to degrade cellulose seemed to be less extended, and only around 20% of isolates from *Pseudoalteromonas* and *Alteromonas* spp., and some strain of *Vibrio* spp., exhibited this ability. In the case of pectin, positive results were only found in around 9% *Pseudoalteromonas* spp. isolates, and some *Alteromonas* and *Vibrio* spp. strain (Fig. 2). None isolate from *Marinomonas* spp. showed the ability to degrade cellulose or pectin, and no strain of Marine bacteria showed any degradative ability on any assayed substrate. Some isolates from *Pseudoalteromonas* and *Alteromonas* spp., and *Glaciecola mesophila*, *Agarivorans albus* and *Shewanella* spp., were also agarolytic (data not shown). Assayed isolates from *Neptunomonas* sp. and *Sulfitobacter pontiacus* did not exhibit degradative potential against any of the three assayed substrates.

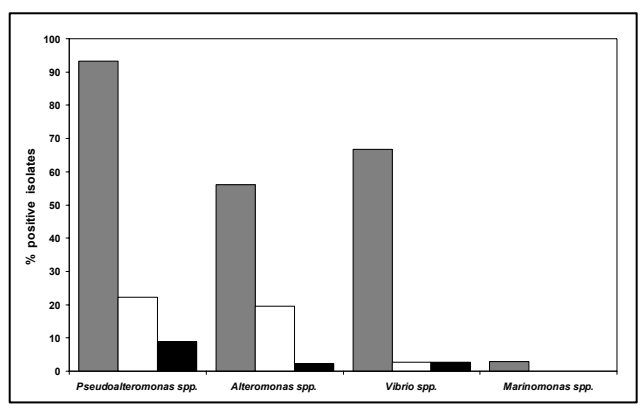

Fig. 2. Number of isolates that gave positive results for degradation of starch, cellulose or pectin, on Marine Agar plates supplemented with each substrate at 1% final concentration. Starch, grey; cellulose, white; and pectin, black colour.

3.3. Enzymatic activities by APIZYM system

Data on the percentage of positive tests in the APIZYM system are shown in Fig. 3. The highest numbers of positive results were exhibited by strains of *Pseudoalteromonas* spp. (a mean of near 7 positive tests) and *Alteromonas* spp. (around 5), whereas the lowest numbers corresponded to isolates from the group of not identified "Marine bacteria" (an average number of positive tests lower than 4).

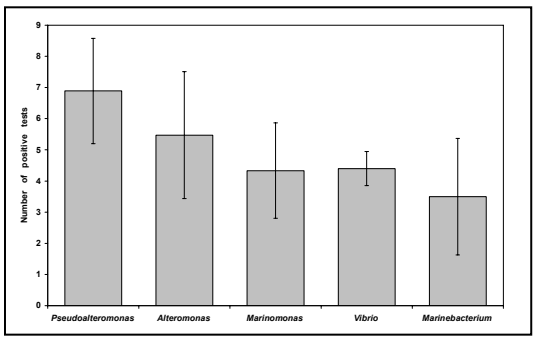

Fig. 3. Average positive results in APYZYM system for all isolates from each bacterial genus recovered from *P. oceanica* meadows. Bars represent the standard deviation.

4. Discussion

In the present work we have studied enzymatic activities of a collection of isolates recovered from several *P. oceanica* meadows at different seasons. The most abundant genera found in association with seagrasses were *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Marinomonas* and not identified Marine bacteria. The strains showing a greater enzymatic potential, as measured both by qualitative and semi-quantitative methods, were those belonging to *Pseudoalteromonas* and *Alteromonas* spp. Isolates from *Vibrio* spp. exhibited mainly amylase activity but most of them were unable to hydrolyze cellulose or pectin. However, those few *Vibrio* strains that showed pectinase and/or cellulase activities, that are less frequent than amylase ones, have been selected for a pathogenicity study on *P. oceanica* plants, as some *Vibrio* species have been related with diseases in aquatic organisms [14, 15].

The genera *Pseudoalteromonas* and *Alteromonas* contain numerous species, many of them associated with marine eukaryotic hosts [16, 17], and many producers of biologically active compounds [12, 18]. As a first approach to assess the ecological significance of the presence of these isolates in *P. oceanica* meadows, we have evaluated the enzymatic potential of the strain collection recovered from seagrasses. The fact that most *Pseudoalteromonas* spp. isolates exhibited amylase activity is an indicative of its potential; *Alteromonas* spp. did not showed so high percentage of amylase-positive isolates, although the values were superior to 50%. Moreover, near 20% of *Pseudoalteromonas* and *Alteromonas* spp. strains were able to hydrolyze cellulose, and some of them even pectin. The results suggest that some bacteria could take advantage of these enzymatic abilities when are in contact with *P. oceanica*, since starch, cellulose and pectin are plant components. Starch is stored in the rhizome of *P. oceanica* during spring and summer, whereas hemicellulose or pectin and cellulose are present in the cell wall of seagrasses (which has no protein, lignin, or lipid) [19]. Furthermore, a possible relationship between plant component degradation and certain APIZYM positive tests is also now under investigation. All those strains from *Pseudoalteromonas* spp., *Alteromonas* spp. or even other less frequent species associated to *P. oceanica* (*Shewanella* spp., *Glaciecola mesophila* and *Agarivorans albus*), exhibiting more than one of the assayed enzymatic activities on plates, are under study at present for evaluating the nature of their interaction with seagrasses.

The production of bioactive compounds by the genera and species selected has already been reported [20], and many of our isolates also have this ability. It is possible that these important enzymatic activities involves a benefit for bacteria in its habitat, seagrasses meadows, having some effect on the aquatic plants, maybe making them more susceptible to any other stress factors. In some cases, antibacterial compounds could protect host from pathogenic microorganisms [21]. These possibilities are being studied, after this work on enzymatic potential of isolates. In conclusion, these results let us to advance in the knowledge of bacterial microbiota associated with *P. oceanica*, with the objective of deeping into its ecological significance in seagrasses ecosystem.

Acknowledgements Work supported through project "Conservación de Praderas Submarinas: Causas de la regresión y efectos sobre las Funciones en el Ecosistema" from the Fundación Banco Bilbao Vizcaya (BBVA) of Spain. M. Ordax thanks MEC for a predoctoral fellowship. Dr. E. Marco-Noales thanks MEC (Programa INIA/CC.AA) and Agroalimed foundation for her contracts. The authors thank B. Águila for technical assistance.

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Development of new primer systems for the detection of the polyphosphate kinase gene in activated sludge

Lysann Mehlig*, Frank Ludwig, Martin Eschenhagen, and Isolde Röske

Department of Microbiology, Dresden University of Technology, D-01062 Dresden, Germany

*Corresponding author: Lysann.Mehlig@mailbox.tu-dresden.de, Phone: +49 351 46336169

An enzyme which regulates the metabolism of phosphate is the polyphosphate kinase (*ppk*). Therefore, it is an important criterion for the identification of polyphosphate accumulating bacteria (PAO) in activated sludge. With the published primer systems it is currently impossible to detect the *ppk* gene simultaneously from different microorganisms.
The aim of this study was the development of new primer systems for the acquisition of a wide range of *ppk* sequences. The specific amplification was tested by cloning and sequencing. As an outcome a large diversity of *ppk* sequences was detected. In addition differences in the composition of the *ppk* sequences in samples of the activated sludge of the investigated plants were shown by using the terminal restriction fragment length polymorphism analysis (T-RFLP).

Keywords EBPR, polyP, ppk, activated sludge, T-RFLP

1. Introduction

The enhanced biological phosphorous removal (EBPR) is a common technology in wastewater treatment. Nevertheless it is largely unknown which microbial species are mainly responsible for this process [1]. The involved bacteria (PAO) are able to store phosphate as polyphosphate in high amounts, much more than they need for their own growth.

The ability to store polyphosphate (polyP) is phylogenetically widespread. It has been found in bacteria, fungi, protozoa, plants and mammalian cells, but its physiological importance remained mostly unexplored [7]. In bacteria the metabolism of phosphate is essential for the regeneration of energy and inorganic phosphate. In this process the enzyme polyphosphate kinase (Ppk) is responsible for the generation of polyphosphate. It catalyses the transfer of the terminal phosphate of ATP to the growing chain of polyphosphate [2,3,4,5]. Based on the central function of the *ppk* in the polyphosphate metabolism this enzyme represents an important criterion for the identification of polyphosphate accumulating bacteria (PAO).

In first investigations with published *ppk* primer systems [3,4,5,6] only a small diversity of *ppk* sequences in activated sludge samples of different municipal wastewater treatment plants were posted. Otherwise an enquiry of database and alignments has shown that this enzyme belongs to a very heterogeneous gene family. Therefore, only a minor diversity of *ppk* sequences was detectable by the published primer systems.

In this study we developed new primer systems for a wide range of *ppk* sequences. The specifity were tested by cloning and sequencing. Thereby a large diversity of *ppk* sequences was detected. Using the new primer systems in combination with T-RFLP significant differences in the composition of the *ppk* sequences in the activated sludge of the investigated plants was shown.

2. Material and methods

2.1 Sludge samples

Activated sludge was sampled from eight municipal wastewater treatment plants with different tech-niques for phosphorous removal out of the aeration basin (Elsterwerda, Bad Liebenwerda, Frankenberg, Hainichen, Arnsdorf, Mittweida, Niederwiesa and Kaditz, Germany). Table 1 displays the operational parameters.

Table 1 Operational parameters of the investigated municipal wastewater treatment plants. AS: activated sludge process; N: nitrification; D: denitrification; EBPR: enhanced biological phosphorous removal; CP: chemical precipitation.

Investigated plant	Mode of operation	Type of wastewater	Sludge age [d]
Elsterwerda	AS, N, D, EBPR	municipal / dairy	18
Bad Liebenwerda	AS, N, D, EBPR	municipal	20
Niederwiesa	AS, N, D, EBPR	municipal	38
Mittweida	AS, N, D, EBPR + CP	municipal	20
Frankenberg	AS, N, D, EBPR + CP	municipal / industrial	21
Hainichen	AS, N, D, CP	municipal	16
Kaditz	AS, N, D, CP	municipal	12
Arnsdorf	AS, N	municipal	50

2.2 Molecular techniques

Genomic DNA was extracted from activated sludge using the FastDNA Spin Kit for Soil (BIO 101, Carlsbad, USA). The isolated DNA was stored at -20°C.

The developed *ppk* primers amplify a *ppk* gene fragment with a length of approximately 1100 bp. Amplification was carried out with 100 to 500 ng of genomic DNA from activated sludge. All PCR amplifications were done by using the reaction mixture HotMasterMix (Eppendorf, Hamburg, Germany) in a final volume of 25 µl, containing 10 pmol of non-degenerated primers (M13f, M13r) or 200 pmol of degenerated primers (ppk1_FW, ppk1_RW, Table 2). The PCR was performed in a thermocycler (mastercycler gradient, Eppendorf) using the following conditions: initial denaturation step at 95 °C for 2 min followed by 38 cycles of 95 °C for 30 s, 55°C for 45 s and 68 °C for 2 min, and a final extension step at 68°C for 10 min. The amplified DNA fragments were observed by agarose gel electrophoresis in 1.5 % agarosegel. Five microliters of each amplification mixture and 1 µl the molecular mass marker (100 bp DNA Ladder Plus, peqlab, Erlangen, Germany) were subjected to agarose gel electrophoresis and ethidium bromide (1 mg/l) staining. The amplified DNA fragments were visualized by UV illumination.

Table 2 Primers used in this study.

Primer	Sequence	Reference
ppk1_FW	AAY YTI GAY GAR TTY TTY ATG GT	this study
ppk1_RW	TTI KYI TSY TCR TCR AAI CKI GC	this study

To generate DNA libraries PCR products of tree WTP´s (Elsterwerda, Bad Liebenwerda, Niederwiesa) were purified using the E.Z.N.A.© Cycle-Pure Kit (peqlab) and cloned into vector PCR2.1-TOPO (Invitrogen, Karlsruhe, Germany). The amplification products with the primers M13f and M13r were used for restriction fragment length polymorphism (RFLP). Two restriction enzymes, *Rsa*I and *Msp*I (Fermentas, St. Leon-Rot, Germany) were used for the digestion (15 µl PCR product, 2 µl enzyme, 2 µl buffer, 1 µl distilled water). The digested DNA was loaded on a 1.5% agarose gel, separated by electrophoresis, stained with ethidium bromide, visualized and photographed under UV-light.

For sequencing the DTCS Quick Start Master Mix (Beckman Coulter, Krefeld, Germany) and the automatical sequence system CEQ 2000 (Beckman Coulter) were used. The clone sequences were compared with the NCBI (National Centre for Biotechnology Information) database.

For T-RFLP analysis *ppk* fragments were amplified with the primers ppk1_FW (fluorescently labelled with Cy5) and ppk1_RW. The amplification products were purified with the E.Z.N.A..© Cycle-Pure Kit (peqlab) and digested with the restriction enzymes *Msp*I (CC/GG) or *Rsa*I (GT/AC) (Fermentas). The PCR products (8 µl) were digested in a total volume of 10 µl, containing 1 µl of enzyme and 1 µl of buffer for 6 h according to the manufacturer's instructions.

The digested PCR products were precipitated by centrifugation (15.500 x g, 4°C, 1 h) after addition of sodium acetate (0.3 M final concentration) and 2 volumes of cold ethanol and an incubation step at -20 °C for at least 30 min. The recovered DNA was mixed with a length standard and analysed with the automatically sequencing system CEQ 2000 (Beckman Coulter).

3. Results

3.1. Development of *ppk* primer systems

In first molecular biological investigations with already published ppk primer systems [3,4,5,6] we only found a small diversity of different ppk sequences in activated sludge samples of eight Wastewater Treatment Plants (WTP) with different techniques for phosphorous removal (Tab. 1). These primer systems are developed mostly for specific bacterial groups. A development of new primer systems for the analysis of the ppk diversity in activated sludge becomes necessary.

The development of our new ppk primer systems is based on an amino acid alignment of known ppk-sequences available by the National Centre for Biotechnology Information (NCBI) (Fig. 2).

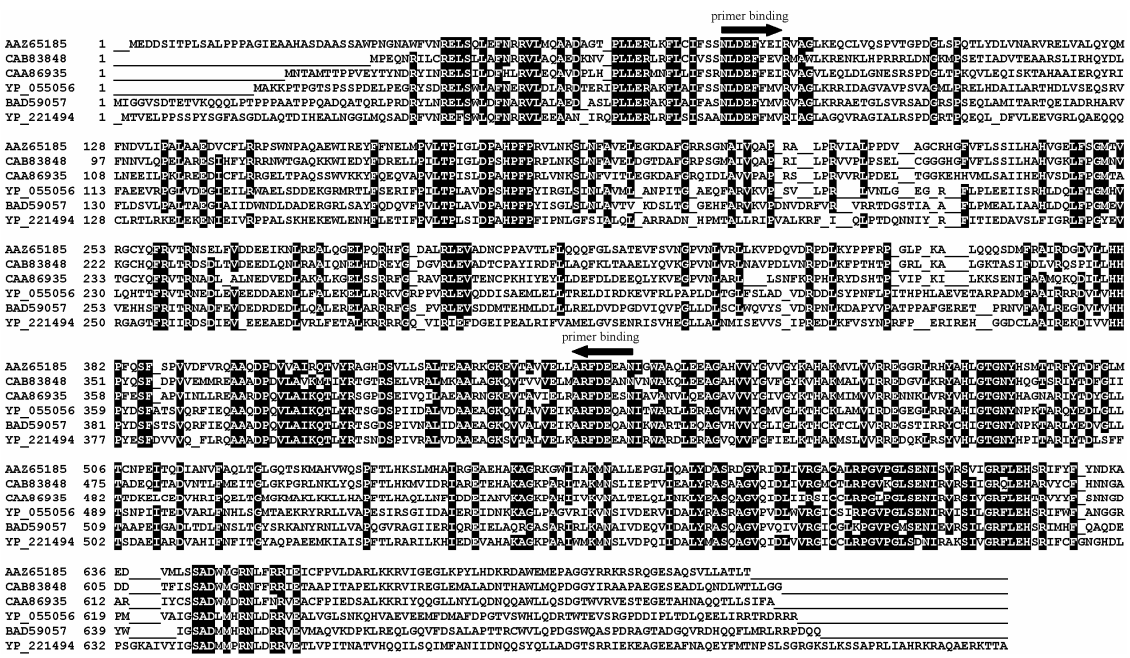

Figure 1 This alignment of the amino acid sequence of the *ppk* of different potential PAO´s indicates the affiliation to a very heterogeneous gene family. The black marked blocks show the homologous areas in the amino acid sequences of the *ppk* of different microorganisms. AAZ65185, protein from *Ralstonia eutropha*; CAB83848, protein from *Neisseria meningitidis*; CAA86935, protein from *Acinetobacter* sp.; YP_055056, protein from *Propionibacterium acnes*; BAD59057, protein from *Nocardia farcinica*; YP_221494, protein from *Brucella abortus.*

Based on this alignment the heterogeneity of the *ppk* gene family is clearly proved. The high variability of the amino acid sequences (Fig. 2) and furthermore the degeneracy of the genetic code reveals a high heterogeneity in the nucleotide sequences.

Therefor we used the primer binding sites NLDE**** (forward primer) and ARFDE*** (reverse primer) for an amplification of a *ppk* sequence fragment. The variable regions in the amino acid sequence of the forward and reverse primers are marked by the asterisks.

Based on this situation a development of just one primer system for all *ppk* sequences was not possible. Due to the high heterogeneity several degenerated primer systems were developed which can prove various known *ppk* gene sequences of potential PAO with different phylogenetic origins. In this paper we only present the results of one primer set.

3.2.Detection of *ppk* gene in activated sludge

A PCR product could be received from every sample by using the new primer system. Therefore the primer system with the widest spectrum of acquisition and degeneration was used in the following studies.

To check out the specificity the obtained PCR products were cloned. Altogether 152 clones from different municipal wastewater treatment plants were analysed by RFLP. Out of these clones 71 restriction patterns could be differentiated.

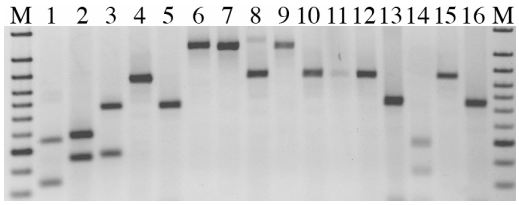

Figure 2 RFLP patterns of 16 clones. M, size marker; 1 to 16, differentiated clones.

The clones with different restriction patterns were used for sequencing. A huge diversity within the ppk sequenceswas found. Figure 4 represents the phylogenetic tree of these sequences which exhibited different RFLP pattern. The diversity suggests that the primers are able to detect an ample diversity of *ppk* gene sequences.

3.3. Diversity analysis with T-RFLP

This analysis showed significant differences in the fragment spectrum of *ppk* gene sequences of different WTP samples. Every investigated sample was discriminated by an individual pattern (**Fig. 5**). Using PRIMER 5 we designed a tree based on these T-RFLP profiles (data not shown). The dendrogram displayed no dependence between technique for phosphorous removal and the identified composition of the *ppk* sequences. The huge variety of different fragment lengths in the T-RFLP profiles demonstrated the wide spectrum of different *ppk* sequences detectible by just one of the new primer systems.

4. Discussion

In this study we developed new primer systems for the detection and analysis of *ppk* gene sequences in activated sludge samples.

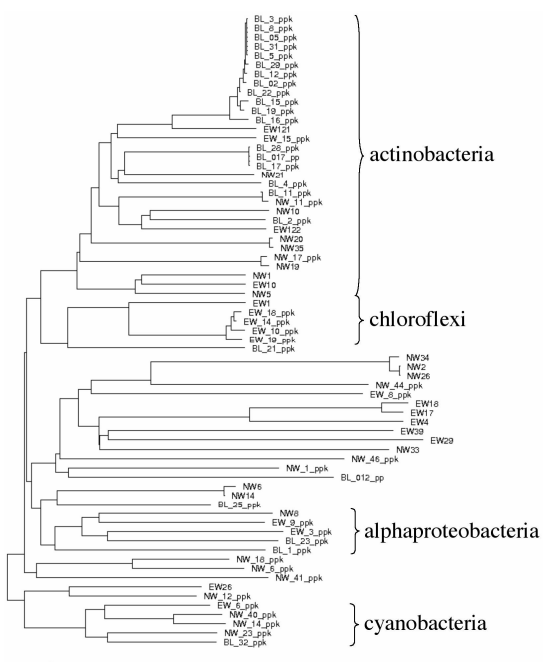

Figure 3 Phylogenetic tree of the obtained *ppk* sequences

The development of new primer systems was nessesary, because the *ppk* belongs to a very heterogeneous gene family and with the published primer systems [3,4,5,6] it was currently impossible to amplify simultaneously *ppk* gene fragments of a wide range of potential EBPR-related microorganisms out of different taxonomic groups.

In contrast to McMahon et. al 2002 who used the NLDE- and the TGNY-region, we have used longer target sites and a different region for the primer design (see Fig. 2). Thereby our primers are more degenerated.

Using our newly developed primer systems we are now able to detect a wide range of *ppk* sequences with only one PCR.

Furthermore, significant differences between the activated sludge samples of the investigated plants could be shown easily by *ppk* specific T-RFLP and RFLP analysis.

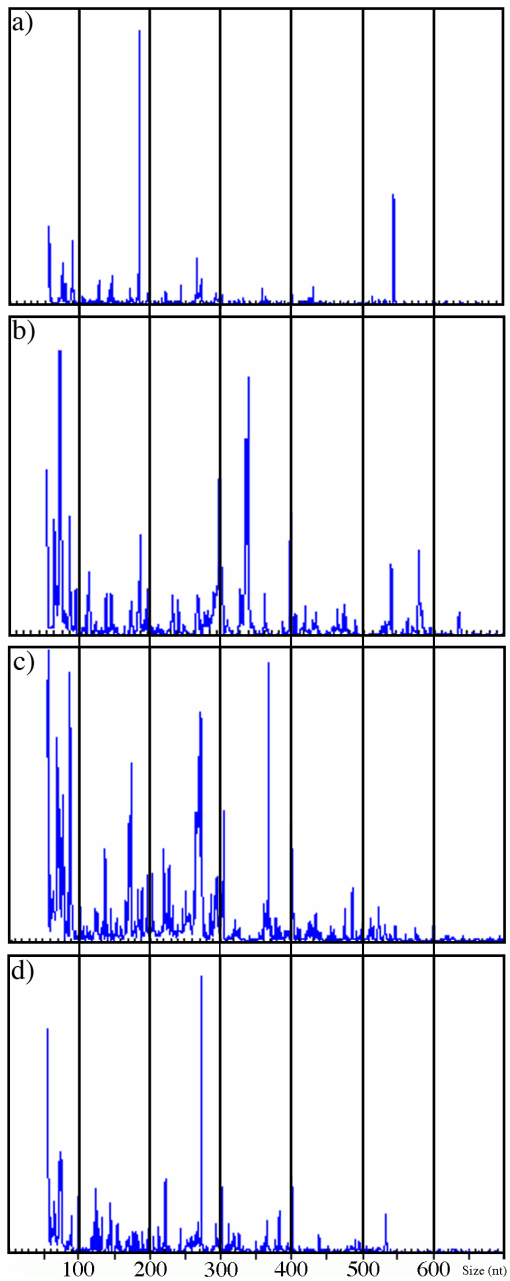

Figure 4 T-RLFP profiles of activated sludge samples from four WTP´s digested with the restriction enzyme *Msp*1, a) Hainichen, b) Elsterwerda, c) Frankenberg d) Kaditz

The results of this report were obtained with primers which offer the verification of the taxonomically most different *ppk* sequences. Therefore, we presented here a first insight into the wide range of different *ppk* sequences. The specifity of the used primers could be confirmed by cloning and sequencing.

To analyze merely the diversity of the genomic *ppk* gene sequences is not enough, because the Ppk is ubiquitary and the diversity reveals no information about the activity and the expression of the polyphosphate kinase during the EBPR-process. Therefore, in further investigations it will become necessary to isolate the mRNA and carry out an mRNA expression study with the activated sludge samples.

Acknowledgements The support by the scholarship program of the German Federal Environmental Foundation (DBU), Osnabrück, Germany (AZ 20007/901) is gratefully acknowledged.

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Effect of Phenanthrene on the Germination, Radial Growth and Chitin and Chitosan production by *Cunninghamella elegans* Lendner

M.C.F. Silva[1,6]; M.Moraes Filho[2,6]; A.M.T. Jara[4,6]; A.C. Silva[4,6]; T.C.M.Stamford[3,6]; G.M. Campos-Takaki [5,6,*]

[1] Post-graduation in Fungi Biology, Phone 51 81 92387606, martacfs@yahoo.com.br;
[2] Post-graduation in Biologic Sciences, Federal University of Pernambuco, Recife-PE, Brazil; Phone 51 81 91527620, marcosamf@hotmail.com;
[3] Integrated College of Patos-(FIP), Patos, PB, Brazil;Phone 5181 96573135; thayzastamford@yahoo.com.br;
[4] Master Course in Development of Environmental Processes, Phone 51 81 21194012; aliciamdpa@yahoo.com.br, antoniocardoso2000@yahoo.com.br,
[5] Department of Chemistry and 6Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Recife, PE, Brazil; Phone: 51 81 21194017; galba_takaki@yahoo.com.br

[*]Email: galba_takaki@yahoo.com.br or gmctakaki@pesquisador.cnpq.br

Our study investigated the effects of phenanthrene on the germination, radial growth, and chitin and chitosan production by *Cunninghamella elegans* (UCP542). Phenanthrene is a polycyclic aromatic hydrocarbons (PAHs) recalcitrant and persistent substance present in petroleum refinery effluents. Chitin and chitosan are a structural component of the cell wall of Mucorales order. The chitin and chitosan have economic value as due to their versatile activities and applications. *C. elegans* was cultivated on synthetic medium for Mucorales, in the condition 2 (glucose 4%, sucrose 1% and sodium chloride 1%), and the condition 8 (glucose 4%, sucrose 2% and sodium chloride 4%), without (control), and with 0.5mg/mL of phenanthrene. The profile of germination of *C.* elegans was evaluated of the sporangioles germination during the intervals of 1 to 6 hours. The radial growth of *C. elegans* was measured the colony diameter during 12 to 96 hours. The chitin and chitosan were extracted by alkali-acid treatments. The results showed that sporangioles germination initiate after 2h of incubation. The higher germination percentage occurred in the conditions 2 and 8, corresponding to 75 %(control) and 61%(treated), respectively. Similar effect was obtained in the radial growth in the presence of phenanthrene. The chitin (53.5%) and chitosan (12.6%) were increased by phenanthrene (0.5mg/L), respectively. The results suggest that phenanthrene has a negative effect on the germination and radial growth, but influenced the production of chitin and chitosan by *C. elegans.*

Keywords Phenanthrene, Cunninghamella elegans, Germination, Growth, carbohydrates

Introduction

Petroleum is a complex mixture of hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) represent an important class of toxic environmental pollutants [1]. The interest in exploiting petroleum-degrading organisms for environmental clean-up has become central to petroleum microbiology, which developing metabolic conditions to biotransformation, biodegradation, and bioremediation of these pollutants [1]. Phenanthrene is a PAHs recalcitrant and persistent substance in petroleum refinery effluents [2]. In the microbial world, hydrocarbons are widely used by bacteria, fungi and algae as a substrate from which to extract energy, and building blocks for cellular structures. The employment of methodologies using fungi to degradation of PAHs as phenanthrene, have been studied extensively. Innumerous fungi have ability to oxidize PAHs using alternative degradative mechanisms as co-metabolism and biodegradation for energy and growth. The capabilities of the microorganisms have resulted in the development of technologies for enhanced petroleum recovery, biorefining and petroleum waste management. Biodegradation is an environmental friendly, having relatively low cost and significant results [3].

On the other hand, innumerous studies have been carried out with microorganisms for to remove toxics compounds in the environment using mechanisms of biosorption [4]. Chitin, is insoluble linear β1,4- linked homopolymer of N-acetyl-D-glucosamine (GlcNAc), and it is the second most abundant polysaccharide, after cellulose. Chitosan is a cationic amino polysaccharide, essentially constituted of β-1,4 D-glucosamine (GlcNAc) linked to N-acetyl-D-glucosamine residues, derived from de-*N*-acetylation of chitin [5]. These polysaccharides are found in a wide range of natural sources, such as crustaceans, insect's annelids, mollusks, coelenterates, and are a common constituent of fungal cell walls. These polysaccharides have great economic value as due to their versatile activities and applications, including as bioremediation and biodegradation [6]. Chitin and chitosan show peculiar properties, such as: biodegradability, biocompatibility, bioactivity, selective permeability, polieletrolic action, chelation, ion exchange properties, antitumor and antimicrobial activity [6], and adsorption

capacity [7,8]. The aims of this investigation were to study the effects of phenanthrene on the germination, radial growth, and chitin and chitosan production by *Cunninghamella elegans* (UCP542).

Materials and Methods

Microorganisms
Cunninghamella elegans UCP 542 (Culture Collection of Catholic University of Pernambuco, Recife, Brazil) isolated from mangrove sediment situated in Rio Formoso, PE, and Brazil. The strain was maintained on Potato Dextrose Agar (PDA) slants at 4°C.

Phenanthrene
The solution was prepared dissolving phenanthrene on acetone in concentration of 2 g/L. The solution was filtrated using a Millipore membrane of 0,45µm and storage in a bottle ambar, at temperature of 5°C.

Culture Medium and Growth profile
The sporangioles of *C. elegans* were harvested from cultures grown for seven days at 28ºC on Petri dishes containing Yeast Malte Agar (YMA) medium. A suspension of cells was prepared and counted 10^8 sporangioles/mL in hematocytometer. For fungal submerse cultivation, 10mL sporangioles suspension (10^7 sporangioles/mL) were inoculated in Erlenmeyer's flasks of 1000mL of capacity, containing 290mL of Hesseltine and Anderson (H&A) medium, modified [9]. Freitas Silva [9] was selected the conditions 2 and 8 from factorial design 2^3, as: condition 2 (glucose 4%, sucrose 1% and sodium chloride 1%), and condition 8 (glucose 4%, sucrose 2% and sodium chloride 4%), in the presence or absence of phenanthrene (0,5 mg/mL). Controls were done using H&A medium containing glucose (4%) in absence of phenanthrene pr presence of phenanthrene. The flasks were incubated at 28ºC, in an orbital shaker, at 150 rpm, during 96 hours. After this period the mycelia were harvested, washed twice in destilled and deionizer water, using silkscreen nylon membrane (120 F) for filtration, and were submitted to freeze dried process. The biomass was maintained in a vacuum dissecator until constant weight.

Evaluation of Germination
The germination was realized removing 10^7 cells/ml of sporangioles to Erlenmeyers of 250 mL of capacity, containing 50mL of H&A culture medium modified, and controls and treated with phenanthrene (0,5 mg/mL) were used. The Erlenmeyer's flasks were incubated at orbital shaker (150 rpm) at 28ºC. The experiments were realized in triplicate. Aliquots of 40 µl were removed per hour after inoculation and 200 sporangioles were counted, utilizing a light microscope. The quantification of germinated sporangioles was made estimating the proportion between germinated and non germinated cellules per hour.

Radial Growth Rate
C. elegans was grown during 48h, in YMA medium, and aseptically was cut discs (5mm diameter) the periphery of the colony, and transferred to the center of Petri dishes containing 20 mL agar H&A medium, added or no of phenanthrene (0.5mg/mL. Petri dishes were incubated in the absence of lights. The radial growth was evaluated by measurement of colony diameter; express in millimeter each 6h, until 48h [10].

Chitin and Chitosan Extraction
The extraction of chitin and chitosan involved deproteinization using 2% w/v sodium hydroxide solution (30:1 v/w, 90ºC, 2 h), separation of alkali-insoluble fraction (AIF) by centrifugation (400*g* 15 min.), extraction of chitosan from AIF under reflux (10% v/v acetic acid 40:1 v/w, 60ºC, 6 h). The separation of crude chitin was done by centrifugation (4000*g* 15 min.), and precipitation of chitosan by adjustment of pH 9.0. Crude chitin and chitosan were washed on a coarse sintered-glass funnel using distilled water, ethanol and acetone, and air-dried at 20ºC [5,7,9,11].

Infrared Espectroscopy (Deacetylation Degree-DD%)
The degree of deacetylation for microbial chitin and chitosan were determined using the infrared spectroscopy and using the absorbance ratio A1655/A3450 and calculated according to the following equation:
A (%) = (A1655/A3450) x 100 / 1.33

Two milligrams sample of fungal chitin and chitosan, which had been dried overnight at 60°C, under reduced pressure were thoroughly blended with 100mg of KBr, to produce 0.5mm thick disks. The disks were dried for 24h at 110°C under reduced pressure. Infrared spectrometer was recorded with a Bruker 66 Spectrometer, using a 100mg KBr disks for reference. The intensity of maximum absorption bands were determined by the baseline method.

Statistic Analysis

The data were analyzed for significance using the Student´s t-test and chi-square test using STATISTICA program version 6.0 of Statsolt Inc., USA. All experiments were carried out in triplicate and the results are expressed as mean ± S.D.

Results and Discussion

The figure 1 showed the effects of sodium chloride, glucose, sucrose and phenanthrene on germination of C. *elegans* sporangioles. The tube germinative was formed at 6 hours of incubation for the experimental conditions 1(glucose 4%, sucrose 1%, sodium chloride 1%) and 2 (glucose 4%, sucrose 2%, sodium chloride 4%), in absence of phenanthrene (controls), corresponding to 60% of the germination. The results showed the inhibition of phenanthrene on the germination process of *C. elegans*. The effect of the polycyclic aromatic hydrocarbons-HAPs on the germination is not observed in the literature. The radial growth of *C. elegans* in presence or absence of phenanthrene in different concentration of sodium chloride, and added of glucose and sucrose is showed in figure 2. The growth in the controls and conditions 2 and 8 started after 12h of incubation. However, in the presence of phenanthrene it is a late process, only initiated after 24h of incubation.

However, we compared that effect of phenanthrene with the use of the pesticide 2,4-D, in concentration of 50.25 and 134% promoting the sporulation in *Cercospora rodmanii*. The authors were attributed this phenomenon, to a defense mechanism of the fungus [10]. Studies were observed in Micromycetes fungi grown in the presence of fluoranthene in 0.001, 0.01 and 0.1g/L concentrations, and decreased the mycelia growth, when increased the toxic concentration of the compound in the medium. The authors were observed the presence of sterile mycelia and discoloration of the colonies in some species by fluoranthene. Researches with anthracene in the concentration of 0.1g/L induced to 95% of the increase of the growth rate. On the other hand, the growth of *Doratomyces stemonitis* and *Cylindrocarpon destructans*, in presence of anthracene, were inhibited in concentration lower of 0.01g/L; *Sporormiella australis* and *Sporotrix cyanescens* were strongly inhibited by anthracene concentration of 0.1 and 1g/L [4].

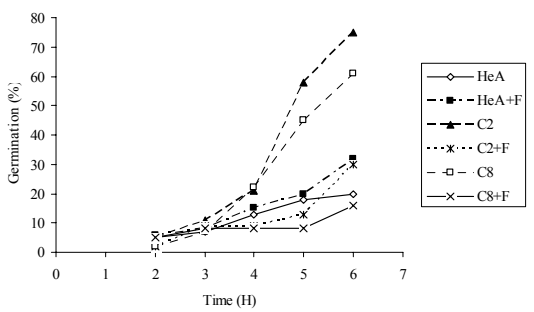

Fig. 1 Germination of esporangioles by *C. elegans* medium H&A control or trated glucose, sucrose, NaCl and phenanthrene.

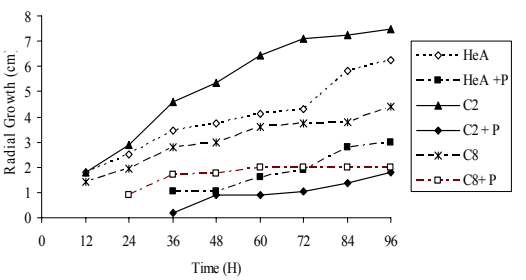

Fig. 2 Radial Growth de *C. elegans* in medium H&A control or trated glucose, sucrose, NaCl and phenanthrene.

The figure 3 showed the percentage of chitin and chitosan extracted from *C. elegans* biomass in the different cultural conditions. The highest production of chitin (53.5%) was obtained in the presence of H&A medium control with 0.5mg/mL of phenanthrene. However, the chitosan increase of the percentage in the condition 2, when was treated with 0.5mg/mL of phenanthrene. The production of chitin and chitosan obtained in these studies showed superior results [7]. In the present study it was demonstrated that chitin and chitosan polysaccharides can be used as substrate in co-metabolic conditions for environmental contamination with PAH. Studies on relationship between the naphthalene mineralization rate and salinity in sediments showed dependence of salinity condition. Those results suggested the presence of sites exhibiting a lack or inhibition of the mineralization in the range of salinities, and preference of the microorganisms to the less saline percentage. In those studies with *C. elegans* the hyper saline condition showed that rates of hydrocarbon metabolism decreased when increasing the salinity range (3.3 to 28.4%), and the behavior of cytoplasmatic membrane. Those results were attributed to the increasing of the cytoplasmatic membrane permeability, reduction of enzymatic metabolism, and corroborated with the literature [12].

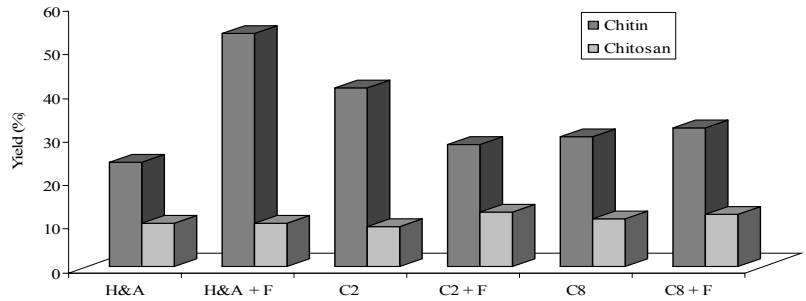

Fig 3 Chitin and Chitosan produced by C. elegans UCP 542 and grown for 96 hours after the pré-inoculum, in medium H&A, varying the glucose, sucrose, NaCl and phenanthrene(0.5mg/mL).

Deacetylation degree (%DD) is an important parameter associated with the physical-chemical properties of chitosan cationic properties. In the present study chitin and chitosan obtained from *C. elegans* grown in the selected conditions (H & A and 2, both treated with phenanthrene) showed 10% DD and 80% DD, respectively. The results described by Franco et al [7], Freitas Silva et al [9] and Stamford et al [11] reported as higher deacetylation degree of chitosan from fungi between 80 to 90%DD. New approaches developed here, we understanding of the diverse physiologies and metabolic tools of the *C. elegans* employ to access, degradation, internalize, and consumption of aromatic polycyclic hydrocarbons (HAPs) is initiated with cytoplasmatic membrane alteration of permeability. In addition, the varied approaches of that microorganism employ to mitigate potentially toxic effects on environment contaminated with HAPs3.

Acknowledgements The authors are grateful to CAPES, CNPq, FINEP for the financial support, and UNICAP for the use of laboratories.

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Effect of Pyrene on the growth of *Rhodotorula sp*

P.H. Campos Marinho[1,3], P. Mendes de Souza[2,3], M.A. Barbosa de Lima[3], A. Elesbão do Nascimento[3] and G.M. Campos Takaki[*,1,2,3]

[1] Programa de Pós-Graduação em Ciências Biológicas - Universidade Federal de Pernambuco, Recife, PE, Brazil
[2] Programa de Pós-Graduação em Biologia de Fungos - Universidade Federal de Pernambuco, Recife, PE, Brazil
[3] Núcleo de Pesquisa em Ciências Ambientais - Universidade Católica de Pernambuco, Rua Nunes Machado, 42, Bloco J, Boa Vista, CEP 50050-590, Recife, PE, Brazil

[*]E-mail: galba_takaki@yahoo.com.br

Among the yeasts, species of the genus *Rhodotorula* can use polycyclic aromatic hydrocarbons (PAHs) as the sole carbon and energy source. The present study was carried out in order to evaluate the effects of different pyrene concentrations, 0.25mg/mL, 0.5mg/mL and 1mg/mL on the growth of *Rhodotorula sp* grown in Yeast Mold Agar (YMA) and Yeast Mold Broth (YMB) media. Significant differences had been observed in the cellular viability, maximum growth rate ($\mu_{esp.}$) and generation time (Tg) according to the concentrations used and to the intervals of growth time. According to the results obtained in this work it is possible to suggest that the inhibitory effect of the pyrene reflects the physiological state related to the cellular stress in presence of the xenobiotic in the culture medium.

Keywords Cellular Viability, Pyrene, *Rhodotorula*

1. Introduction

The interaction of yeast and polycyclic aromatic hydrocarbons (PAHs) is relatively well documented. However, little information about the resistance of yeast to the pyrene has been published. The studies of the interactions between the yeasts and polycyclic aromatic hydrocarbons (PAHs) have a high scientific value. Among the fungi, yeast microorganisms are the most exploited scientifically to have physiological and biochemical mechanisms common to all eukaryotes for the study of basic cellular phenomena, and thus serve as an excellent model for the study of many important aspects of the biology of eukaryotes [1].

The pyrene is one of the most abundant polycyclic aromatic hydrocarbons (PAHs) found in the environment. Is an ubiquitous and persistent pollutant having toxic, mutagenic and carcinogenic properties [2]. Moreover, the yeasts are the most numerous fungi found in polluted environments. Some species of the various yeasts genera have the ability to metabolize these xenobiotic compounds, as a source of carbon and energy. However, high concentrations can inhibit microbial growth. Members of the genus *Rhodotorula* are able to grow, for example, in liquid media containing aromatic, with or without co-substrates, and can transform any liquid hydrocarbon present in the medium [3,4].

The yeasts exist in nature in a wide variety of substrates and environments. It is know that the microorganisms show great flexibility in their adaptation to different environments. This adjustment results certainly of its features biochemical, physiological and genetic. Moreover, it is known fact that environmental conditions can influence the habits of many microorganisms in a positive or negative [5].

Thus, studies on the relationship between a particular microorganism and its environment are essential to the characterization that determines its metabolic versatility to adapt, and that otherwise, also determines the characteristics observed in relation to the potential of this group of organisms. This study aimed to investigate the effect of pyrene on the different stages of growth of yeast *Rhodotorula sp*.

2. Materials and Methods

2.1. Microorganism

It was used the isolate of *Rhodotorula sp* (UCP 1003) kindly supplied by the Culture Collection of the Nucleus of Research in Environmental Sciences (NPCIAMB) of the Universidade Católica de Pernambuco, Brazil. The microorganism was maintained in Yeast Mold Agar (YMA) medium slants under refrigeration at 4°C.

2.2. Culture Media

2.2.1. Maintenance Medium

For the maintenance was used Yeast Mold Agar (YMA) medium described by CIRIGLIANO & CARMAN [6], consisting of the following nutrients: Yeast Extract (0.3%), Malt Extract (0.3%), D - Glucose (1%), Peptone (0.5%), Agar (1.5%) and Distilled Water q.s.f (100mL). The components were solubilized and the pH was adjusted to 5.8. The medium was sterilized at 121°C, during 20 minutes.

2.2.2. Growth Medium

It was used the medium Yeast Mold Broth (YMB), also described by CIRIGLIANO & CARMAN [6], containing the same nutrients of YMA medium, without agar.

2.3. Microbiological Methods

2.3.1. Pre-Inoculum

The *Rhodotorula sp* was grown in Yeast Mold Agar (YMA) medium at 28ºC during 24 hours, then, a loopful of the stock cultures were transferred to Erlenmeyer flasks of 500mL capacity, containing 250 mL of Yeast Mold Broth (YMB) medium and incubated aerobically for 24 hours, on a rotary shaker, to 150 rpm (revolutions per minute), at 28°C. A culture contained 10^7 cells/mL was used to initiate growth in the treated and control medium using a 15% v/v of the inoculum. Samples were taken throughout the cultivation period and the following characteristics were evaluated: maximum growth rate ($\mu_{esp.}$), generation time (Tg) and cellular viability, monitored by cells counts on Neubauer Hemocytometer.

2.3.2. Conditions of Cultivation - Treatment with Pyrene

It used the Pyrene in concentrations of 0.25mg/mL, 0.5mg/mL and 1mg/mL. The hydrocarbon, in concentrations above, was added to Erlenmeyer flasks of 500mL, containing 250mL of YMB medium and then sterilized. Aliquots of 15mL of pre-inoculum were added to flasks and cultures were incubated during, 72 hours, at 28°C in orbital shaker (150 rpm). Each culture was made in triplicate; three flasks not received the pyrene, corresponding to the control culture.

2.3.3. Growth Curves

The growth curves of *Rhodotorula sp* were accompanied during the intervals of 8, 12, 16, 20, 24, 48 and 72 hours of cultivation. The monitoring was conducted by the cells counts in Neubauer Hemocytometer.

2.3.4. Maximum Growth Rate ($\mu_{esp.}$) and Generation Time (Tg)

The kinetic parameters maximum growth rate ($\mu_{esp.}$) and generation time (Tg) were evaluated. Both parameters were determined according to the formula described by PIRT [7]. For the specific growth rate was used the equation: $\mu_{esp.} = (LnX - LnX_0) / (T - T_0)$; Where: X = maximum number of cells; X_0 = initial number of cells, T = time when the maximum number of cells is achieved and T_0 = initial time. The generation time (Tg) was determined by the formula: $Tg = Tg = Ln2 / \mu_{esp.}$

2.3.5. Cellular Viability

The analysis of cellular viability of the control and treated cultures of *Rhodotorula sp* was conducted by platting of 0.1 mL of each culture, properly diluted in phosphate buffered saline, pH 7.2, in the YMA. Thereafter, the plates were incubated for 28°C during 24 hours. After this interval, the colonies were counted each plate was made in triplicate and aliquots collected at intervals of 8, 12, 16, 20, 24, 48 and 72 hours of incubation. The number of colonies was given by the average of the values obtained in the three plates.

2.4. Biochemical Tests

2.4.1. Determination of pH

The determination of culture media pH, along the cellular growth, was performed by potentiometry over the growth of microorganism in the intervals of 8, 12, 16, 20, 24, 48 and 72 hours of cultivation. The pH values variation, at the intervals, corresponded to the average of three measures.

2.4.2. Glucose Consumption

The determination of glucose was performed by the Oxidase Peroxidase method - Enzymatic Colorimetric (Kit Celm), which is based on the enzymatic oxidation of glucose by the enzyme glucose oxidase. Samples of cultures supernatant, collected at the intervals of 8, 12, 16, 24, 48 and 72 hours of cultivation were used for the determination of glucose consumption during growth. The readings were made in Spectrophotometer Digital Spectronic, Model Genesys 2.

3. Results

Figure 1 shows the growth curves treated cultures with pyrene and control. It was possible to observe that control and treated cultures exhibited an initial phase of adaptation, Lag phase of approximately 8 hours, followed by the exponential, Log phase increase in growth which is extended up to 48 hours of incubation. After this period the stationary phase was achieved by cultures.

The analysis of figures 1A, 2A, 3A and 4A shows the pH decrease over time in cultures treated and control. At the same time, the total glucose consumption occurred during the first 8 hours of cultivation.

Analyzing the same figure, it was observed that the addition of pyrene in the concentrations tested had influence on the growth of the cultures of *Rhodotorula sp*. The growth curves pattern of pyrene treated cultures was different compared to control culture. Meanwhile, the evaluation of the results obtained for treatment with pyrene showed that the concentrations of 0.25mg/mL, 0.5mg/mL and 1mg/mL induced inhibition of the growth. Moreover, the culture treated with 0.25mg/mL of pyrene showed no difference when compared to control sample. The observation of the figure pointed out that 0.5mg/mL and 1mg/mL of pyrene induced the highest growth inhibition.

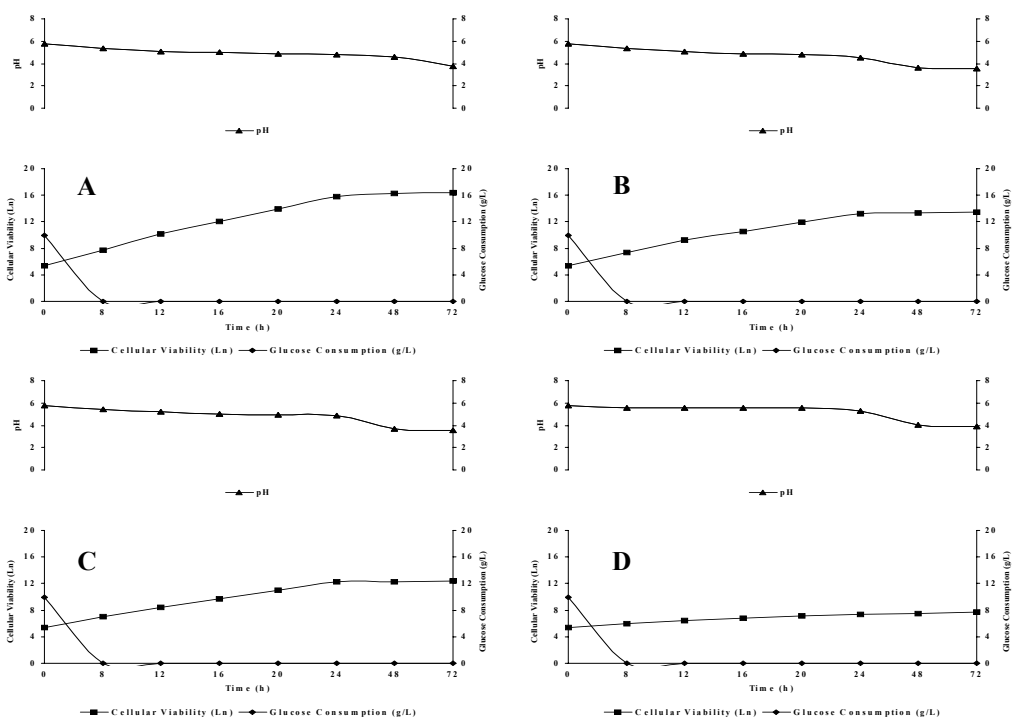

Figure 1. Effect of Pyrene on cellular viability of *Rhodotorula sp*. A. Control; B. 0.25mg/mL; C. 0.5mg/mL and D. 1mg/mL.

Table 1 shows the values of the kinetic parameters studied. It was found that the values of the maximum growth rate and generation time, obtained for the cultures treated with pyrene, with different concentrations, differ from those obtained for the control culture.

Moreover, the treatment of the culture with pyrene in the concentration of 1mg/mL induced the highest inhibitory effect on the growth of the microorganism. It is observed that these values were significantly different from those of control. Also, the maximum growth rate for the culture treated with 1mg/mL was relatively low when compared with other cultures in various stages of growth.

Table 1. Kinetic Parameters of *Rhodotorula sp* grown in the presence and absence of pyrene

Time (h)	Generation Time (T_G)				Maximum Growth Rate (h^{-1})			
	Control	0.25mg/mL	0.5mg/mL	1mg/mL	Control	0.25mg/mL	0.5mg/mL	1mg/mL
8	2.38	2.85	3.62	10.6	0.290	0.243	0.191	0.065
12	1.73	2.19	2.70	7.87	0.402	0.316	0.256	0.088
16	1.66	2.15	2.59	8.15	0.416	0.321	0.267	0.085
20	1.62	2.13	2.50	8.25	0.427	0.324	0.277	0.084
24	1.59	2.13	2.44	8.34	0.434	0.326	0.284	0.083
48	3.08	4.21	4.84	16.2	0.225	0.165	0.143	0.043
72	4.55	6.13	7.14	21.6	0.152	0.113	0.097	0.032

4. Discussion

The understanding of the action mechanisms of polycyclic aromatic hydrocarbons (PAHs) on the growth of different microorganisms, and in particular yeasts, has been the subject of several studies. The yeasts and filamentous fungi have diversified metabolism that provides them the use of a variety of nutrients in different environmental conditions [8, 9].

The results obtained in this work suggest that the yeast, *Rhodotorula sp*, present cellular viability being able to grow in the medium containing pyrene, in different concentrations tested, but it was not observed any effect of this compound on stimulating the growth of the microorganism. This is possibly linked to the fact that different microorganisms can behave so differently in respect to the presence of pyrene in the culture medium. The growth of a microorgasnism is complex and highly coordinated process, which involves many cellular components that accompany the development and cell differentiation. The basic trend of the growth is exponential, but this may be modified by nutritional and physical limitations of the environment. In general, the microbial metabolism represents the balance of appropriate combinations of components of the growth medium, chemical and physical properties that lead to growth and synthesis of constituents essential to the organism [10, 11, 12].

It can be observed from the results obtained, that the treated cultures behaved in a way other than in relation to the concentration of the compound added to the culture medium of *Rhodotorula sp*. The concentration of 1mg/mL of pyrene caused a drastic reduction in the growth of microorganism, when compared with the concentrations of 0.25mg/ml and 0.5mg/mL.

Thus, one can suggest that the results obtained in this work on pyrene effects at different stages of growth of *Rhodotorula sp*, probably are associated with the toxicity of xenobiotic and the composition of the culture medium. Aromatic compounds probably exert a toxic effect at the level of the membrane, as it follows from the high correlation between the toxity and hydrophobicity. Also, the cellular response to different concentrations of polycyclic aromatic hydrocarbons, or any other compound, even if it is not toxic, is possibly linked to the particular physiological characteristics of each organism studied [12,13].

5. Conclusions

The effect pyrene in the concentrations tested, 0.25mg/mL, 0.5mg/mL and 1mg/mL decreased the growth of yeast. The hydrocarbon presented a toxic effect on the *Rhodotorula sp*, translated by reducing the growth in the different growth phases, with this mainly effect at 1mg/mL, compared to the control culture. The values obtained showed a good correlation with the growth and viability of the microorganism, reflecting the physiological state of stress to which the yeast was submitted.

Acknowledgements: This work was carried out with the financial support of CAPES, FINEP / CTPETRO, CNPq and Universidade Católica de Pernambuco, Brazil.

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Effect of the introduction of an anaerobic phase on the protozoa community of an SBR used for biodecolorization of an azo dye

N. Dana Lourenço[*,1] **J. Maggiolly Novais**[1], and **H. Maria Pinheiro**[1]

[1] IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Universidade Técnica de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

[*]Corresponding author: e-mail: nidia.lourenco@ist.utl.pt, Phone: +351 218419065

The development of the activated sludge technology has led to several investigations on the importance of protozoa and other consumers in the sewage treatment processes. The Sludge Biotic Index (SBI) was accepted as an objective index to estimate the biological quality of sludge in the aeration tanks of activated sludge plants based on the microscopic analysis of protozoa, in terms of its density and diversity. In the present work, this method was used to assess the biological quality of the sludge from a Sequencing Batch Reactor system treating an azo dye containing, simulated textile effluent in 24-hour cycles with 8 hours of aeration preceded by 13 hours of non-aerated reaction. The aim was to correlate SBI to treatment efficiency. After 50 days of operation the protozoa progressively disappeared and at the end of the studied period (100 days) the SBI values had decreased from 9-10 to 4. This was however not accompanied by decreases in the COD or color removal yields.

Keywords SBI; protozoa; SBR; non-aerated/aerated; textile effluent

1. Introduction

The protozoa display a wide variation in size, from 1μm to several centimeters [1], reflecting adaptations at the cell level, and their systematization is, like for many other eukaryotes, based on morphological traits [2]. In spite of being unicellular organisms, they can display multicellularity, where each individual colony develops from a single cell through repeated cell divisions, and also some complex morphological adaptations. One typical example of this complexity is the external morphology of the ciliate Euplotes, including bundles of individual cilia with which the organism can walk on surfaces and also membranelles in the mouth with which it filters suspended food particles from the surrounding water [1].

Specific communities of ciliated protozoa can be found in high density in all types of aerobic biological treatment systems. The development of the activated sludge technology led to several investigations on the importance of protozoa and other consumers in the sewage-treatment processes. In activated sludge systems the microfauna can be divided in two main groups, i.e., the decomposers and the consumers. The decomposers correspond to the bacteria and the fungi, which directly decompose the organic matter present in the wastewater and the consumers correspond to the heterotrophic flagellates, the ciliates and the small metazoans, which consume the dispersed bacteria and other organisms.

Several relationships were encountered between the ciliated species present in the activated sludge plant and different variables such as the operating parameter settings, the effluent quality data, and the incoming sewage quality and flow rate [3]. Based on these relationships, various methods using microscopic analysis of the microfauna were developed to predict the quality of the treated effluent and to assess the general performance of the treatment system [3, 4]. The Sludge Biotic Index (SBI) was proposed as an objective index to estimate the biological quality of the sludge from aerated tanks of all types of activated sludge plants based on the microscopic analysis of protozoa, in terms of its density and diversity [5]. In this method the protozoa are subdivided into three keygroups, namely, the free-swimmers, the crawlers and the attached sessile. The SBI values, on a scale of 1 to 10, are determined by a two-way table, taking into account not only the dominant keygroup and its density, but also the total number of taxonomic units identified in the microfauna of the examined activated sludge and the number of small flagellates. The SBI values can then be further translated into four activated sludge quality classes, a higher class representing a lower quality level.

An activated sludge Sequential Batch Reactor (SBR) is potentially an appropriate system for azo dye biodegradation in textile wastewaters, since it can be manipulated in order to provide an anaerobic phase, during which azo dyes are reduced to the corresponding aromatic amines, and an aerated phase where the amines can be aerobically mineralized [6]. However, the use of the SBI for quality monitoring in such systems has not been validated.

In the present study, the SBI method was used to assess the biological quality of the sludge from an anaerobic/aerobic (SBR) system treating a simulated, azo dye containing textile effluent, working in 24-hour cycles with 8 hours of aeration preceded by a 13-hour non-aerated reaction period. Namely, the possibiliy of

correlating SBI values to the system's performance in terms of colour and COD removal was investigated, particularly in what concerned the presence of the dye and the introduction of a non-aerated period in the reaction cycle.

2. Methods

2.1 Experimental system

The SBR system was composed of two, 1-liter reactors inoculated with activated sludge taken from a full-scale, continuous activated sludge plant (Beirolas, Loures, Portugal) and acclimatized to a dye-free, simulated textile effluent (hydrolyzed starch derivative, 600 mg COD l^{-1} d^{-1}) during 1 month. After this period, a reactive azo dye solution (Reactive Violet 5, pre-hydrolyzed) was daily injected into one of the reactors (90 mg l^{-1} initial concentration) and the second reactor was used as a dye-free control.

The 24-hour sequential cycles consisted of five discrete periods: fill - 50 min, react - 21 h (mixed react - 13 h, aerated react - 8 h), settle - 1h, draw - 55 min and idle - 15 min. During the acclimalization phase, the whole react period was aerated. Air was supplied through ceramic diffusers by a Hiblow SPP 20GJ-L air pump (Techno Takatsuki Co., Japan) and additional mixing was provided by magnetic stirring (IKAMAG-EOA 3 with the control unit ES 5, IKA-Labortechnik, Germany). Mini-S 660 (Ismatec, Switzerland) peristaltic pumps were used to fill and drain the reactors. The pumping, aeration and agitation functions were computer controlled. The daily purge of mixed liquor was performed manually towards the end of the mixed reaction phase in order to impose a sludge retention time of 15 days in the reactors.

2.2 Analytical methods

Chemical Oxygen Demand (COD) and SBI values were determined for both the dye-fed and the dye-free SBR along the acclimatization period and also during the following experimental period of 100 days. COD was determined according to standard procedures [7]. SBI was determined by means of a two-way table on the basis of keygroups, density (individuals per liter, estimated from the number of individuals counted in each observed sample) and number of taxonomic units identified [5].

Microscopic observation of the microfauna was performed with a light microscope (Olympus CX40, Japan, 100x magnification) using 25 µl mixed liquor samples collected 6-8 hours after the start of the react period. The number of small flagellates was counted along the diagonal of a Fuchs-Rosenthal chamber at 200x magnification. All the visual screenings were repeated twice.

Color removal was quantified in the dye-fed SBR by spectrophotometric analysis (Hitachi U-2000, Japan) of mixed liquor samples, clarified by centrifugation, using a calibration curve based on the absorbance measured at the dye peak absorption wavelength in the visible region (560 nm).

3. Results and discussion

The SBI values calculated for the dye-fed SBR and the color and COD removal yield values obtained in the corresponding 24-hour cycles throughout the experimental period, including the pre-adaptation of the sludge to the base feed without dye, are presented in Fig. 1. The SBI values calculated for the dye-free SBR and the COD removal yield values obtained in the corresponding 24-hour cycles throughout the same experimental period are presented in Fig. 2.

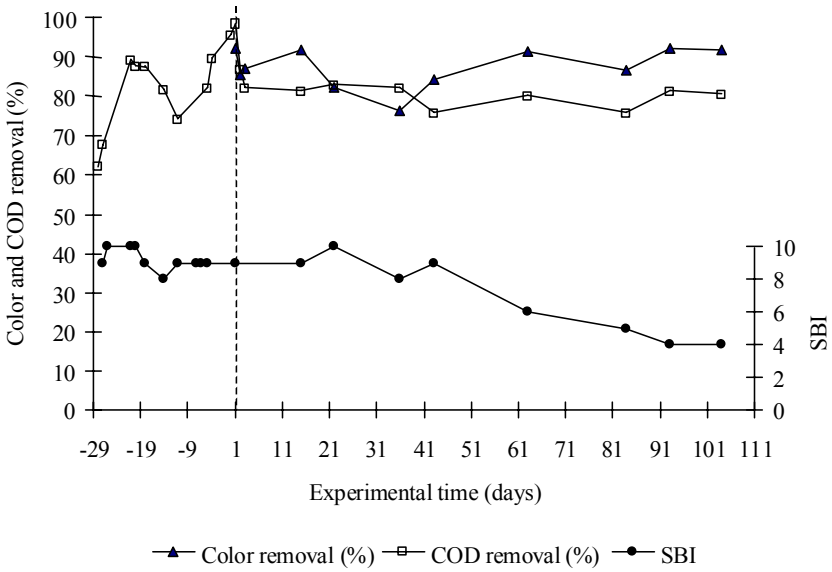

Fig. 1 SBI values calculated for the dye-fed SBR together with the color and COD removal yield values obtained in each 24-hour cycle. The negative-value experimental days correspond to the pre-adaptation of the sludge to the base feed without dye (29 days) and the dashed vertical line indicates the start of dye injection in the SBR, which coincided with the introduction of the non-aerated react period.

During the first 50 days of non-aerated/aerated cycle operation, the SBI values in both the dye-fed and the dye-free SBR were high and a large diversity of microfauna was observed, predominantly from the crawling and sessile keygroups. According to Madoni [5] these results indicated a very well colonized and stable sludge, an excellent biological activity and a very good general performance. However, during the subsequent 50 days of operation, a progressive disappearance of protozoa was observed from both reactors. In this period, the predominant keygroup identified was that of the free-swimming ciliates (*Tetrahymena* sp.), accompanied by the metazoan (*Rotifer* sp.).

Judging from the SBI values mesaured in the second half of the experiomental period, the SBR system would be expected to exhibit a very low efficiency in terms of biological treatment. However, in the present case the decrease in the SBI value was not accompanied by a decrease in the COD removal yield in any of the reactors, and no significant decrease in the color removal efficiency was observed in the dye-fed SBR (Fig. 1). Also, comparing Fig. 1 and Fig. 2 it was observed that the presence of the azo dye had no significant effect on either the SBI values or the COD removal levels. These results suggest that the introduction of an anaerobic phase in the SBR operational cycle, in order to promote decolorization of the textile effluent through azo dye reduction, is not tolerated by the majority of the protozoa. Similarly, the density and diversity of the microfauna observed in an anaerobic/aerobic SBR system treating swine breeding wastewater was previously reported to be significantly lower than that existing in a conventional activated sludge process [8]. However, small free-swimming ciliates such as *Tetrahymena* are reported to be highly tolerant to oxygen depletion and can become the dominant keygroup under these conditions [5]. On the basis of the described observations, it can be concluded that the SBI method cannot be used, in its basic form as a sludge biological quality index, to predict the general performance, in terms of COD removal capacity, of the studied activated sludge bioreactor system.

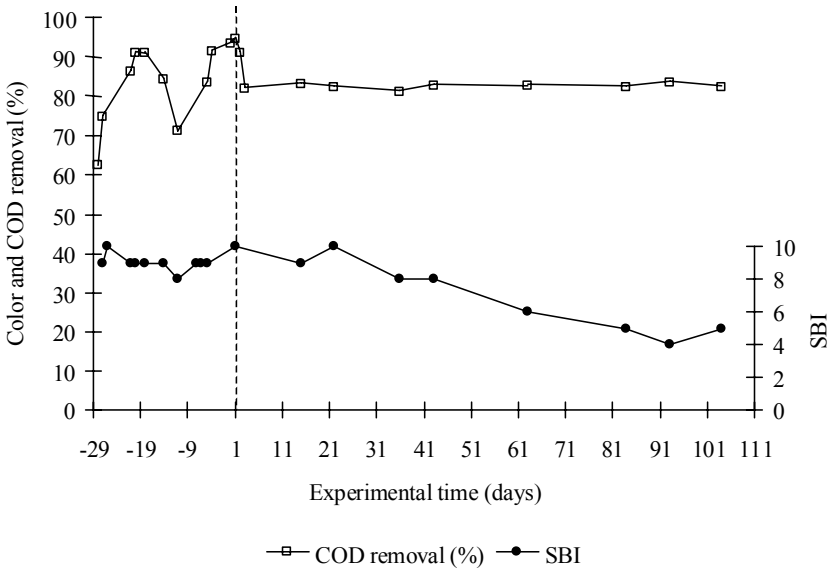

Fig. 2 SBI values calculated for the dye-free SBR together with the COD removal yield values obtained in each 24-hour cycle. All other conditions are as in Fig. 1 and the dashed vertical line indicates the start of dye injection in the dye-fed SBR.

4. Conclusions

A Sequential Batch Reactor (SBR) treating an azo dye containing feed was monitored with the SBI method and the latter value was not significantly affected by the presence of the dye. However, the introduction of a prolongued anaerobic phase in the operational cycle was apparently not tolerated by the majority of the protozoan community initially present in the SBR operated in essentially aerated conditions. In spite of this, COD and color removal yields remained high. Thus, the SBI method could not, in its basic form, be used to predict the performance of this system in terms of its effluent quality parameters.

Acknowledgements This work was supported by FCT (Portugal), contract no. PDCT/AMB/59388/2004.

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Effect of water dilution and nutrient supplements (wood ash, urea and poultry droppings) on biogas production from brewers spent grain

C. O. Onwosi[1,*] and G.U. Okereke[1]

[1] Biotechnology Research Centre, Nnamdi Azikiwe University, P. M.B.5025, Awka, Anambra State, Nigeria.

[*]Coresponding author: email: onwosicoach@yahoo.com; +2348034920577

Owing to the growing concern for alternative sources of energy, the use of brewery spent grains as potential sources of energy (biogas) has been investigated. Brewery derived biowastes (spent grains) were collected from different breweries in southeast Nigeria. These wastes were digested anaerobically in IL laboratory scale digesters over a hydraulic retention time of 14 days (HRT 14) using cow rumen liquor as source of inoculums. An optimization study was carried out by varying some parameters as water dilution and nutrient supplements (wood ash, urea and poultry dropping). The spent grains gave biogas yield of 58-65% methane. Out of the four sets of spent grains used, feed to water ratio of 1:4w/v gave the best biogas yield for three while feed to water ratio of 1:6w/v gave the best yield for one. During nutrient supplementation, highest biogas yield was recorded for wood ash while urea supplements gave the least biogas yield. Increased carbon to nitrogen ratio was found to encourage biogas production. Wood ash increased carbon to nitrogen ratio while urea decreased it. These tests indicated that brewers spent grains can be utilized for biogas production when digested anaerobically and the sludge generated thereafter can provide high quality manure since nitrogen content of the stabilized bio-wastes increased at the end of the digestion as revealed by the proximate analysis.

Keywords biogas; brewers spent grains; HRT; methane; nutrient supplements; manure

1. Introduction

At the start of the new millennium, waste management has become a political priority in many countries. One of the major problems today is how to cope with an increasing amount of primary waste in an environmentally acceptable way. Bio-waste, that is, municipal, agricultural or industrial organic wastes, as well as contaminated soil have traditionally been reloaded in land fills or even dumped in to the open sea or lakes without much environmental concern [1].

The anaerobic digestion of the spent grains (bio-waste) is both a convenient way of reducing pollution as well as a process of energy generation. In today's energy demanding life style, need for exploring and exploiting new sources of energy which are renewable as well as eco-friendly is a must [2]. Spent grains produced by brewery industries are environmental pollutants. One way to tackle the menace of spent grains is to biodegrade them anaerobically with the view to producing biogas. Biogas is a mixture of 53-73% methane, 30-40% carbon dioxide and trace of other gases like nitrogen and hydrogen sulphide. The fuel value of the gas depends on the methane content. Pure methane has a calorific value of 37mJ/m^3 and biogas containing 65% methane would have a calorific value of 24mJ/m^3 [3].

Anaerobic degradation of organic substrates, which is one of the oldest processes used for the treatment of industrial wastes and stabilization of sludge, results in biogas production. This is the usually a relatively slow process since it is carried out by a consortium of microorganisms and depends on various factors like pH, temperature, HRT, C/N, etc [2]. There is also the dried anaerobic treatment of municipal solid waste with the dry anaerobic conversion (DRANCO) processes which produce nitrogen rich residue [4]. The process is an anaerobic solid fermentation for concentrated substrates; it produces energy in form of biogas and humus like end product called humotex [5]. Van der Berg and Kennedy [5] have reported that lack of process stability, low loading rates, slow recovery after failure and specific requirements for waste composition are some of other limitations associated with anaerobic digestion. Therefore there is every need to improve on efficiency of the anaerobic process. This could be achieved by manipulating the various operational parameters, in order to satisfy the nutritional requirements of microbes [8] using chemical additives and by manipulating the feed proportions [7].

Therefore, the objective of this project was to study the effect of water dilutions, urea, wood ash and poultry droppings on biogas production from spent grains collected from different breweries in southeast Nigeria.

2. Experimental

2.1 Feedstock and microbial inoculum

The digester feedstock used were, spent grains collected from four different breweries in southeast Nigeria. The spent grains from these breweries were sun dried and store differently in stoppered polythene containers. The microbial inoculums for the digestion were collected from the rumen of cows slaughtered at a nearby abattoir and strained with cheese cloth [9]. The rumen fluid was chosen because it contained actively growing methanogenic bacteria. Once the culture is introduced into the digester, the decomposition process starts and gas production rate simultaneously increased.

2.2 Anaerobic digester

Experiments were carried out in 1L laboratory scale digester. The digester was operated at a room temperature varying from 26-30^{o}C through the experiment with a consultant HRT of 14 days. The digester set-up consisted of one liter flask stoppered with rubber bungs through which a delivery tube was connected to an inverted burette. 50ml of the inoculum was used to initiate digestion. The methane yield was measured by passing the biogas through inverted column filled with limewater. The limewater absorbed the carbon dioxide and the residual gas volumes (expressed as percentage of the original volumes) were recorded as methane yield.

2.3 Effect of water dilution on biogas production

To determine the effect of water dilution on biogas production, 50g of feed (spent grains) from the four different breweries were placed in sixteen different 1L conical flasks. The spent grains in the different digester flasks were moistened with water to give feed to water ratio of 1:4, 1:6, 1:8 and 1:10 w/v respectively. This was repeated for the four different sets of the spent grains representing different sources.

2.4 Effect of nutrient supplements on biogas production.

Different quantities of dry wood ash were added into four 1L conical flasks, each containing 100g of feedstock (spent grains). A total of sixteen 1L flasks were used for the four different sets of spent grains. In set one digested one, 100g of spent grains without wood ash served as the control. Digester, 2, 3, 4 had spent grain to wood ash ratio of 5:1, 4:1 and 3:1 w/w respectively. This was repeated for set two, three and four of the spent grains and biogas was yield determined by water displacement methods.

The procedures described above were also carried out using poultry droppings and also urea instead of wood ash.

2.5 Analytical method

The following analyses were conducted for spent grains in the four different set of digester before and after 14 days of digestion. Carbon was estimated by taking to be 58% of the volatile matter according to the method of Tinsley and Nowakowski [10]. Total nitrogen was determined by the Kjeldahl method according to Tinsley and Nowakowski [10]. Total phosphorus was determined through spectrophotometer by comparing the absorbance at 420nm with those of the standard solution. Potassium was determined by flame photometry according to method of Allen et al [11].

3. Results and discussion

The total biogas yield of the digester after hydraulic retention time of 14 days (HRT 14) was the highest for all the samples when the feed to water ratio were 1.4w/v (figure 1). But when the feed to water ratio was adjusted to 1:10w/v, the biogas yield was generally low for the four samples studied while the control that was not diluted with water did not produce any biogas. At the dilution of feed to water ratio of 1:4w/v, the biogas production was highest probably it was the best dilution for the digestion because report by Fernando [11] showed that excess dilution does not encourage bacterial cluster formation. This attribute is needed for excellent biogas production. Also Shoed and Singh [12] showed that the dilution ratio of 1:5w/v for water hyacinth and water produced maximum biogas yield as that dilution enabled the biomass to soak the optimum degradation and production of biogas.

Supplementing the bio-waste with ash improved the biogas yield than when supplemented with urea N or poultry dropping. The addition of ash probably increased the carbon content and therefore increased the C/N ratio. On the other hands, the urea supplement reduced the C/N ratio since the addition of urea N increased the N content. The urea supplement reduced the total biogas yield when compared with control that had no urea. When the daily biogas production pattern of the four sets of the spent grains at different nutrient supplements were compared(data not shown), it was found that urea supplements wastes showed greater reduction in biogas yield than poultry dropping supplements wastes. This may be attributed to the fact that the poultry droppings contained some remnant of their unconsumed feed which made it to have higher carbon content than urea. These results agree with those reported by Vermullen *et al.* [13]; they showed that high concentration of N reduced the biogas production rate. This was improved by addition of paper which contributed to increase in C/N ratio. Radhika *et al.* [14] used coconut pit as a source of carbon to supplement cow dung to improve methane production. Sharma *et al.* [15] reported also that the digestion of leaves and straws along with cow dung was generally found to give higher biogas yield than cow dung alone. El shannawi *et al.* [16] combined rice straw, and corn stalks with manure to improve the methane production. Van Buren [17] suggested that this was a higher yield of biogas when carbon comes from different materials rather than one source. This was because the consortium microorganism involved in biogas production attacked the substrate at different rates. Heo *et al.* [18] reported that the adjustment of C/N ratio during digestion could avert any indication of failure such as low pH, insufficient alkalinity, ammonia inhibition and accumulation of volatile fatty acids (VFA). Therefore C and N content should be well regulated for optimal biogas production. There was decrease in phosphorus and carbon content while that of N increased (Table 1). The amount of biogas produced by digestion of the spent grains may have been influenced by the chemical properties of the waste materials. It has been reported by Sathianathan [19] that the difference in the chemical properties of the biowaste control the rate of production of biogas. The reduction in some of the nutrient contents of the spent grains after digestion indicates that they were utilized by biogas producing microorganisms. The increased in N content after digestion is similar to that of Edelman *et al.* [20] that showed that N content of cow manure increased by about 55% and pig manure by about 10%. Also Fernando and Dangoggo [21] reported an increased in N content at the end of the digestion by 16-50% for the various feed stock used. The C/N ratio of the four samples of spent grains used for this study ranged between 7:1-12:1 before digestion but after digestion it become 5:1-10:1 due to increase in N content of the slurry.

Table 1 Proximate analysis of the spent grains before and after digestion

	NUTRIENT CONTENT											
	Before digestion						After digestion					
Sample	%						%					
	P	K	OM	C	N	C/N	P	K	OM	C	N	C/N
1	0.08	0.02	93.54	54.26	5.60	10:1	0.05	0.04	70.17	40.70	6.02	7:1
2	0.10	0.09	60.53	35.11	5.88	6:1	0.06	0.08	72.91	42.29	7.43	6:1
3	0.08	0.05	77.04	44.69	3.64	12:1	0.04	0.05	64.66	37.51	4.34	9:1
4	0.08	0.05	83.92	48.68	3.92	12:1	0.05	0.05	67.41	39.10	4.20	9:1

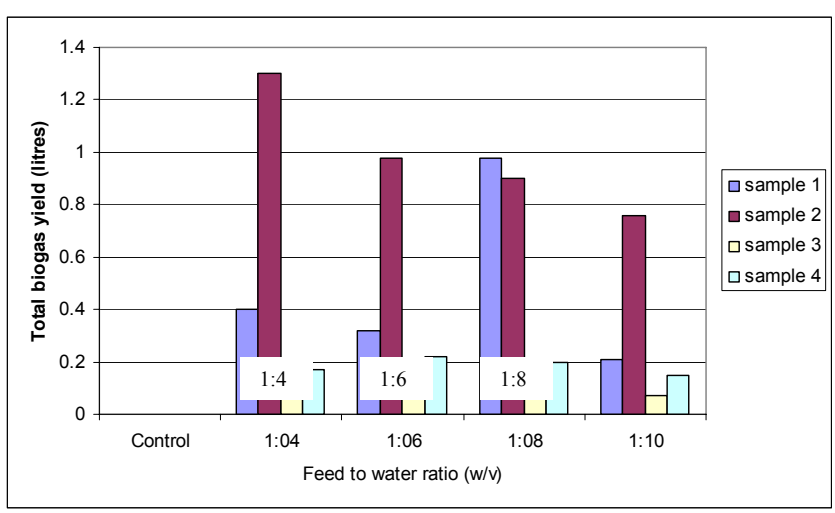

Figure 1 Total biogas yields at different feed to water ratio

4. Conclusion

The results obtained in the study have shown that spent grains when digested anaerobically offer good source of biogas. Under different optimized conditions, the methane yield for samples 1, 2, 3 and 4 were 63%, 65%, 64% and 57% respectively. Therefore, brewers spent grains which are waste produced in the brewery industries can be used in biogas production provided that the digests conditioning were optimized. The sludge obtained after digestion has potential application as rich organic manure because of the increase in N content of the sludge after anaerobic digestion [22].

Acknowledgement The financial support by A.I. Ikeyina and Sons Ltd. is gratefully acknowledged.

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Evaluation of the impact of two aquaculture systems on bacterial communities of the estuarine system Ria de Aveiro

A. Almeida[*,1], **A. Cunha**[1], **L. Santos**[1], **S. Salvador**[2] and **A. Gomes**[2]

[1] CESAM and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
[2] Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

[*]Corresponding author: aalmeid@ua.pt, Phone: +234 370784

The main objective of this work was to determine the influence of two aquaculture systems on bacterial communities of the estuarine system Ria de Aveiro. Two aquaculture systems subjected to different environmental conditions were selected. One, located near the city of Aveiro (Aquaculture A), subjected to some contamination introduced by human wastes and, therefore subjected to chemotherapy treatment; the other located in a clear area distant from the city (Aquaculture B) and where chemotherapy is not used. The studied bacterial groups varied differently along the two systems, in aquaculture B the proportion of bacterial groups was similar near entrance, discharge and in the two tanks, but in aquaculture A the proportion of bacterial groups varied drastically between the entrance and the discharge point of the system. In Aquaculture B it was observed a clear seasonal variation with the highest values of bacteria in May and the lowest in December/January. Contrarily, in Aquaculture A the seasonal pattern of variation was not so clear. The functioning of aquaculture B does not seems to influence the microbiological diversity and water quality of the estuarine system Ria de Aveiro, but the functioning of aquaculture A may strongly affect the microbial communities of the estuarine system. The results of this preliminary study suggest that bacterial abundance in aquaculture B is mainly influenced by seasonal variation. In Aquaculture A, however, some other factors of variation, as chemical treatments of culture water, affect the seasonal pattern of bacterial abundance and bacterial diversity.

Keywords bacterial communities, faecal pollution, aquacultures, estuarine system, impact

1. Introduction

The main biological agents that cause food-borne disease are bacteria, viruses, parasites and to a lesser extent moulds. Parasite-related food safety concerns in aquaculture are limited to a few helminth species, and the hazards are largely focused on communities where consumption of raw or inadequately cooked fish is a cultural habit [1]. Human viral diseases caused by the consumption of fish appear to present a low risk, while viruses causing disease in fish are not pathogenic to man. Bacterial diseases are a major problem in the expanding aquaculture industry. The level of contamination of aquaculture products by pathogenic bacteria will depend on the environment and the bacteriological quality of the water where the fish is cultured. There are two broad groups of bacteria of public health significance that contamine products of aquaculture: those naturally present in the environment - indigenous microflora (e.g. *Aeromonas hydrophila*, *Clostridium botulinum*, *Vibrio parahemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Listeria monocytogenes*) and those introduced through environmental contamination by excreta of domestic animals and/or human wastes – non-indigenous microflora (e.g. *Enterobactereaceae* such as *Salmonella*, *Shigella*, *Escherichia coli*) [2]. It should be noted that non-indigenous bacteria of faecal origin can be introduced into aquaculture ponds via unavoidable contamination by birds and terrestrial animals associated with farm waters.

The main objectives of this work were to evaluate the level of bacterial contamination in two aquaculture systems and evaluate their impact on the water quality of the estuarine system Ria de Aveiro.

2. Materials and Methods

Water samples were collected in two aquaculture systems subjected to different environmental conditions (i.e. different level of human wastes contamination and of chemical drugs addition). One, located near the city of Aveiro, subjected to some contamination introduced by human wastes (Aquaculture A) and, therefore subjected to chemotherapy treatment, and the other located in a clear area distant from the city (Fig. 1) and where chemotherapy is not used (Aquaculture B). Water samples were collected at several points in the two systems: RA (estuarine system, near aquaculture entrance), RE (input reservoir), culture tanks with *Spanus aurata* (tanks 2 and 3) and *Dicentrarchus labrax* (tanks 5 and 7), SG (general discharge), TD (decantation tank) and RS (estuarine system, near aquaculture discharge) in Aquaculture A and RA (estuarine system, near

Fig. 1: Ria de Aveiro with the two aquaculture systems A and B indicated by the arrows.

aquaculture entrance), RE (input reservoir), culture tanks with *Spanus aurata* (tanks 2 and 5) and *Dicentrarchus labrax* (tanks 3 and 7 in aquaculture B), SG (general discharge) and RS (estuarine system, near aquaculture discharge) in Aquaculture B. Water sampling started two hours before low tide and finished around low tide in both systems. Samples were analysed for total bacterial number and for the same specific groups of bacteria. The specific groups of bacteria were accessed by FISH using 16S rRNA target probes [3] in April 2007. The probe Eub338-II-III was used to quantify the most of bacteria belonging to the Domain Bacteria. The bacteria belonging to the non-indigenous *Enterobacteriacea* family and to the *Vibrio*, *Aeromonas* and *Pseudomonas* genera were detected with the specific probes ENT183, VIB572a, AERO1244 and Pae997, respectively. Samples were analysed for bacterial indicators, faecal coliforms (FC) and faecal enterococci (FE) and for cultivable bacteria (CB) on four dates (October 2005, December2005/January 2006, March/April 2006, May 2006). Faecal coliforms and enterococci were enumerated by filter-membrane method using selective culture media, m-FC medium (Difco Laboratories) and m-KF (Difco Laboratories), respectively. Cultivable bacteria were enumerated by pour-plate method using Plate Count Agar (PCA, Difco Laboratories). Statistical analyses were conducted using the statistical software PRIMER version 5.

3. Results

The total bacterial number was around two times higher in Aquaculture A than in Aquaculture B (6.2-11.0 x 10^6 cells mL^{-1}). The most abundant specific group of bacteria in aquaculture A belong to *Enterobacteriaceae*, but in aquaculture B the abundance of this group was similar to that of Vibrio (Fig. 2). The studied bacterial groups varied differently along the two systems, in aquaculture B the proportion of bacterial groups was similar near entrance, discharge and in the two tanks, but in aquaculture A the proportion of bacterial groups varied drastically between the entrance and the discharge point of the system. In general, water of Aquaculture B showed a lower faecal contamination than that of Aquaculture A (Figs. 3). In Aquaculture B it was observed a clear seasonal variation with the highest values of faecal bacteria in May and the lowest in December 2005/January 2006 (Figs. 3). Contrarily, in Aquaculture A the seasonal pattern of variation was not so clear (Figs. 3). In fact, cluster and MDS analysis of the data showed that FC, FE and CB were grouped according sampling date in Aquaculture B but not in aquaculture A (Figs. 4 and 5).

In both aquacultures, bacterial abundance was, in general, higher at RE station (input reservoir), than at SG station (general discharge). However, the concentration of FC, FE and CB were higher at RS station (estuarine system), reaching, frequently, values 1 to 2 folds higher than that observed at SG station.

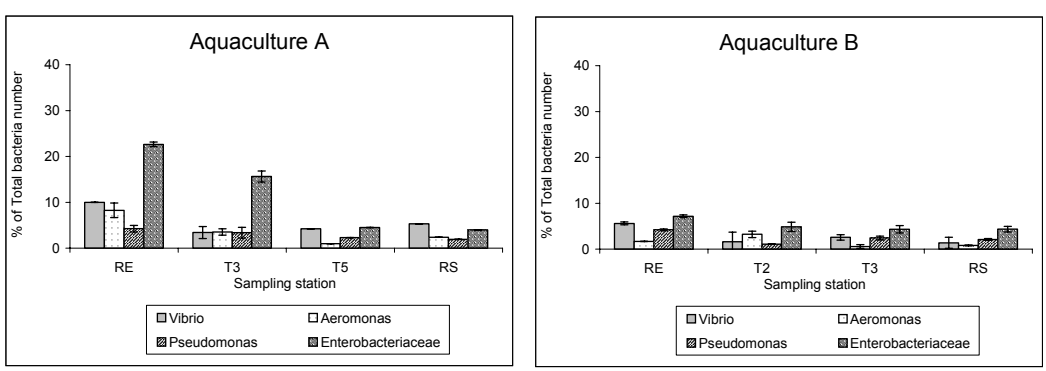

Fig. 2: Percentage of non-indigenous *Enterobacteriacea* family, *Vibrio*, *Aeromonas* and *Pseudomonas* genera detected by FISH in the two aquaculture systems.

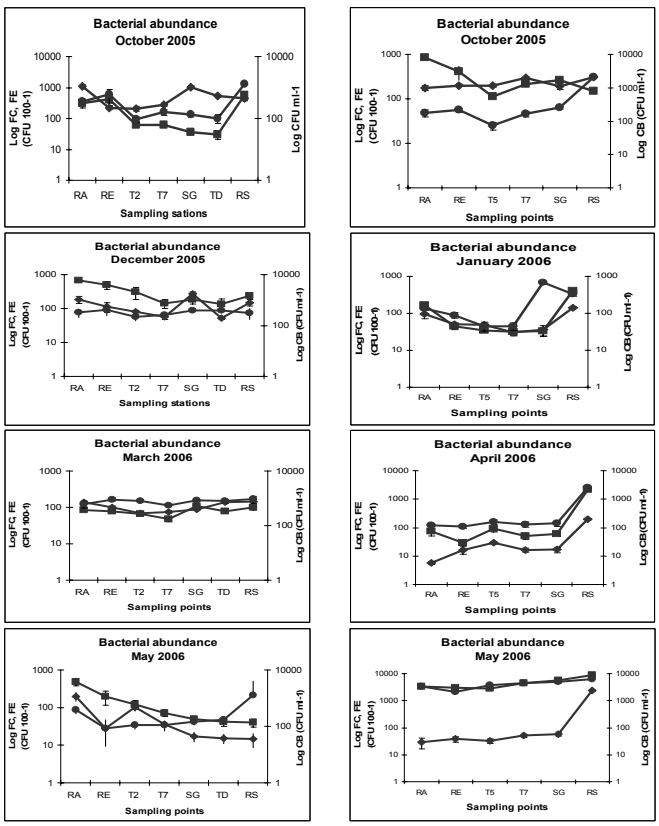

Fig. 3: Seasonal variation of faecal coliforms (FC), faecal enterococci (FE) and cultivable bacteria (CB) in the two aquaculture systems. —■— FC, —◆—

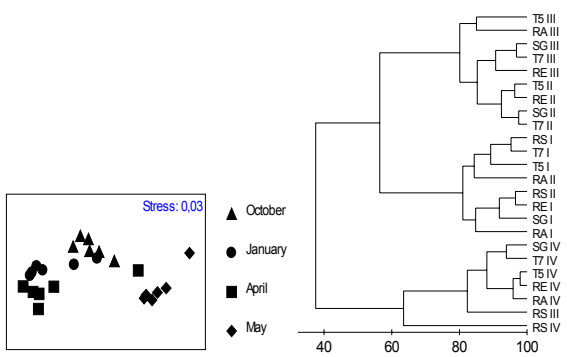

Fig. 4 Cluster and MDS analysis for FC and FE in Aquaculture B.

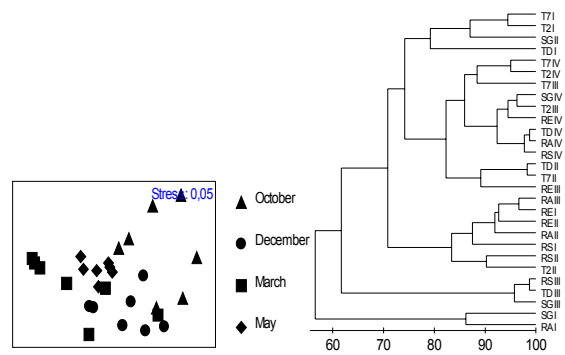

Fig. 5 Cluster and MDS analysis for FC and FE in Aquaculture A.

4. Discussion

The results of this preliminary study suggest that bacterial abundance and diversity in Aquaculture B is mainly influenced by seasonal variation. In Aquaculture A, however, some other factors of variation, as chemical treatments of water, affect the seasonal pattern of bacterial abundance and diversity. The functioning of Aquaculture A, contrarily to Aquaculture B, seems to influence the microbiological water quality of the estuarine system Ria de Aveiro.

As in Aquaculture A the total bacterial number was constant along the system, but the density of the minor specific groups decrease very much between the entrance and the discharge point in the estuarine system, the chemical treatment applied in this system is selective in relation to the bacterial community.

In both aquacultures the levels of faecal bacteria and of cultivable bacteria were significantly higher at the discharge point in the estuarine system. However, bacterial density was, in general, higher at the input reservoir than at the general discharge. The high values registered near the aquaculture water discharge can be attributed to tide effect. We started collecting water 2 hours before low tide, but we finished sampling at the discharge point around low tide, when the levels of bacteria in the Ria de Aveiro are, in general, 2 times higher [3].

As it is well known, in aquaculture systems, in general, some antibiotics are administrated as a preventive measure, but in case of bacterial infections it is used an increased dose of those chemicals. This fact can explain the different pattern of variation observed between the data obtained for the bacterial groups and the data obtained for the three indicators of faecal/organic pollution, since these determinations were done in different dates. This means that the effect of aquaculture systems functioning on bacterial community of the surrounding waters is an intermittent phenomenon and, as obliviously, depends on the quality of the culture water.

Acknowledgements This research was supported by CESAM (Centro de Estudos do Ambiente e do Mar) of Univerty of Aveiro. The authors acknowledge the collaboration of the two aquaculture staffs in water sampling.

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Evaluation of the microbial diversity in anaerobic reactors fed with sucrose applied to hydrogen production

Sandra I. Maintinguer*, Bruna S. Fernandes, Iolanda Duarte, Kátia Saavedra, M. Bernadete A. Varesche

Department of Hydraulics and Sanitation, School of Engineering in São Carlos (EESC), University of São Paulo (USP), Av. Trabalhador São-carlense, 400, 13550-590, São Carlos, SP, Brazil

*Corresponding author: e-mail: mainting@sc.usp.br, Phone: +55 16 3373-8358

The goal of this work was to purify and keep cultures of hydrogen-producers anaerobic bacteria of seed sludge after heat-treatment. Serial dilutions were carried out after the heat-treatment. This purified culture was used in the inoculation of anaerobic batch reactors (2L). The reactors were fed with different sucrose concentrations (in triplicate) and kept at 37°C, with pH equal to 5.5 and headspace filled with He (99.99%) in the following conditions: (1) 629.8 mg sucrose/L; (2) 1183.7 mg sucrose/L; (3) 1815.6 mg sucrose/L and (4) 4127.6 mg sucrose/L. It was used 20% (v/v) of the seed sludge from the cellular purification. The biogas produced in the reactors was analyzed in gas chromatographer and it was verified that the reactors did not produce methane. The efficiencies of the sucrose conversion to H_2 to the conditions (1), (2), (3) and (4) were equal to 15% (1.2 mol H_2/mol sucrose), 20% (1.6 mol H_2/mol sucrose), 15% (1.2 mol H_2/mol sucrose) and 4% (0.3 mol H_2/mol sucrose), respectively. The generated volatile fatty acids were acetic and butyric in all of the anaerobic reactors and also iso-butyric, and iso-valeric acids to the conditions (3) and (4). At the end of the experiment, values of pH of 4.1; 4.0; 4.6 and 3.8 were observed to the conditions (1), (2), (3) and (4), respectively. The biological hydrogen gas production occurred due to bacterial consortia that were present in the studied conditions and confirmed by molecular biology analyses were due the presence of *Enterobacter cloacae*, *Clostridium* sp. and *Clostridium acetobutyricum* recognized as H_2 and volatile acids producers.

Keywords Hydrogen production; sucrose, fermentation, *Clostridium* sp.

1. Introduction

Cleaner energy sources have been applied in order to satisfy the global energetic demand. The hydrogen gas generated in the wastewater treatment by biological processes can be used as an alternative energy source. In this way, the knowledge about the hydrogen-producers microorganisms is fundamental to the development of alternative and cleaner sources of energy production. The hydrogen fermentative production can be facilitated with methanogenesis inhibition, once the methanogenic archaea cells use hydrogen in the anaerobic biological processes. The heat-treatment of the seed sludge associated with the pH control has been applied in the selection of hydrogen producer's bacteria as *Clostridium* sp. These bacteria, endospores-formers, are tolerant to high temperatures and adverse environmental conditions.

In order to study the production of hydrogen and the microorganisms involved in the process, this experiment was evaluated in an anaerobic batch reactors fed with different sucrose concentrations.

2. Material and Methods

2.1 Seed sludge

The seed sludge was taken from granulated sludge of an UASB reactor treating swine wastewaters. It was heat-treated at 90°C for 15 min to inactivate hydrogen consumers and to harvest spore-forming anaerobic bacteria such as *Clostridium* sp [1]. Serial dilutions were carried out after the heat-treatment. This purified culture was used in the inoculation of anaerobic batch reactors. The modified medium [2] , beyond sucrose (1800.0 mg/L), urea (40.0 mg/L), peptona (1000.0 mg/L) and 1.0 mL of vitamin solution [3]. The vitamin solution was composed of *p*-aminobenzóic acid (40 mg/L) and biotine (10 mg/L).

2.2 Reactors operation

Batch anaerobic reactors (2L) were used (in triplicate) to four different conditions of sucrose in concentrations (mg/L): (1) 629.8; (2) 1183.7; (3) 1815.6 and (4) 4127.6 in medium modified [2]. The inoculum (20% v/v) was

composed of reactivated cells, after purification. The HCl or NaOH was added to adjusting pH in 5.5, added 1000 mg/L of peptone and 1mL of vitamin solution. The reactors had been submitted to the He (99.99%), during 20 minutes, at 37^{o}C, without agitation. The conditions (1), (2) and (3) were confectioned with 1500 mL of liquid volume and 500 mL of headspace. The condition (4) was confectioned with 1000 mL of liquid volume and 1000 mL of headspace in the same conditions of the previous conditions.

2.3 Analytical methods

Hydrogen content in biogas was determined by a gas chromatography (GC 2010 Shimatzu), using a thermal conductivity detector and air as a carrier gas. The temperatures of injector, detector and column were kept 30°C, 200°C e 230°C, respectively. Organic acids present in the liquid phase were measured using gas chromatography (HP GC 6890/FID) [4]. Sucrose concentration was determined by the colorimetric method [5]. The volatile suspended solids (VSS), Chemical oxygen demand (COD) and pH were carried out according [6].

2.4 Analysis cellular growth

The cellular growth was based on optical density at 600nm (OD_{600}) [6]. The volatile suspend solids (VSS in g/L) of the biomass was proportional to the optical density at 600 nm and calculated following equation (3):

$$VSS = 2.6691 \text{ (absorbance 600nm)} – 0.0095 \quad (3)$$

2.5 Microbial analysis

The analysis of the microbial structure obtained at the end of the process was performed. The PCR/DGGE technique was applied with primers to the *Bacteria* Domain: 968FGC and 1392R [7]. All the strands of the purified PCR products were sequenced with primers EUB968f by ABIPRISM Big terminator Cycle Sequencing Kit (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Search of the GenBank database was conducted using the BLAST program.

2.6 Experimental analysis

The experimental data had been adjusted for average values gotten of reactors by software Microcal Origin® 5.0. The maximum specific hydrogen production rate had been gotten through the adjustment not linear sigmoid of the Boltzman function.

3. Results and Discussion

The anaerobic reactors had different behaviors, with production of hydrogen gas (Figure 1 and Table 1). For the conditions (1) and (2) not observed lag phase of production of H_2. The conditions imposed in that case favored the production of H_2 in the early hours of operation. However, conditions (3) and (4) there were phase lag in the production of H_2. This fact was related to higher concentrations of substrates that inhibited bacterial growth and therefore the sucrose decomposition with H_2 generation of reactors. In anaerobic reactors of the condition (3) were higher H_2 production. To conditions (1) and (2), the intermediate products were generated acetic acid, butyric and hydrogen gas. For the conditions (3) and (4) was also observed, iso-butyric and iso-valeric acids, but in low concentrations. The generation of acetic acid increased gradually at the beginning of the experiments, and when butyric acid was detected in the reactors was obtained, concomitantly, production of high hydrogen gas. The increase in the proportions of butyric acid was directly proportional to the consumption of sucrose, and consequent production of hydrogen gas. Khanal *et* al. (2004) observed generation of acetic, butyric and propionic acids to pH 5.0 in reactors fed with sucrose (1500 mg/L) with production of 240 mg H_2/g DQO [8].

There was no production of methane in anaerobic reactors, confirming the efficiency of the heat-treatment of seed sludge and imposition of the initial pH 5.5. The combination of these two factors caused the inhibition of bacteria and archaea consumers of H_2.

Two routes degradation of sucrose may occur in fermentations processes for the production of H_2: (1) sucrose decomposition generating acetic acid and (2) sucrose decomposition generating butyric acid. In this experiment were two routes of degradation; but the conversion of sucrose to acetic acid was favored at the beginning of the experiment and the butyric acid in the end.

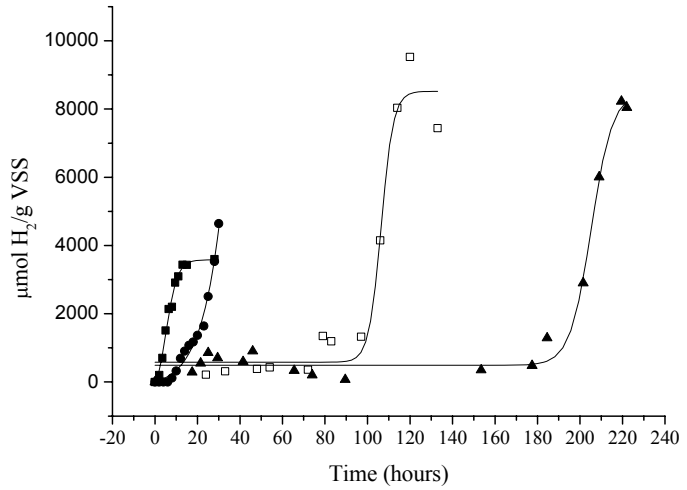

Figure 1 - Hydrogen production in anaerobic reactors to conditions: (■) 629.8 mg sucrose/L; (●) 1182.7 mg sucrose/L; (□) 1815.6 mg sucrose/L and; (▲) 4127.6 mg sucrose/L.

The DGGE profile of the different fed sucrose concentrations did not show significant variation in the standard profiles of the *Bacteria* Domain (Figure 2). The Table 2 shows the results of the sequence affiliation. The fermentative bacteria known for producing hydrogen include *Enterobacter*, *Bacillus* and *Clostridium* species [9]. The bands 1 and 3 showed affiliation with *Enterobacter cloacae* (98% and 97%, respectively). The bands 1 and 3 were considered the same population. The band 2 showed 98% affiliation with *Burkholderia cepacia* and was present for the condition (1), (2) and (4). This bacterium commonly found in soil, water, roots of plants and associated with the mycelium of the fungus [10]. The bands 4 and 5 showed affiliation with *Clostridium* sp. (90% and 98% respectively). The band 6 showed affiliation with *Clostridium acetobutyricum* (96%). These bands are repeated, to a lesser intensity, in other conditions studied. That evidence was expected because of the conditions imposed experiment provided the permanence of Gram positive rods, spores formers; it is characteristic of some bacteria producing hydrogen gas [11]. Several factors could contribute to the maintenance of species of *Clostridium* in anaerobic reactors: addition of peptone in nutrition media [12]; heat-treatment of seed sludge associated with pH 5.5 enriched such populations [11].

Table 1 - Results gotten for the four studied conditions

Parameters analyzed	**(1) 629.8 mg/L**	**(2) 1183.7 mg/L**	**(3) 1815.6 mg/L**	**(4) 4127.6 mg/L**
Sucrose consumed (%)	80.4	87.2	83.0	70.3
VSS (g/L)	0.26	0.30	0.45	0.34
Period (h)	9.5	20	96	201.5
pH (experiment end)	4.1	4.0	4.6	3.8
Operation time (h)	28	96	202	222
Acids (mg/L)				
Acetic	131.5	128.6	537.0	204.7
Butyric	1.2	3.3	252.5	281.4
Iso-butyric	0	0	11.2	0.6
Iso-valeric	0	0	23.2	5.6
H_2 (µmol/L)	2683.2	4576.2	16187.5	2454.5
Period (h)	28	48	129	219.5
Maximum specific sucrose consumption				
(mmol sucrose/L.h)	0.26	0.16	0.11	0.11
Period (h)	3.5 – 5.0	8 - 12	72 - 92	162 – 186
Maximum specific H_2 production rate				
(µmol H_2/g VSS)	2147.3	3201.6	10059.5	2663.1
Hydrogen yield	15%	19.8%	15%	3.8%
(mol H_2/mol sucrose)	1.2	1.6	1.2	0.3

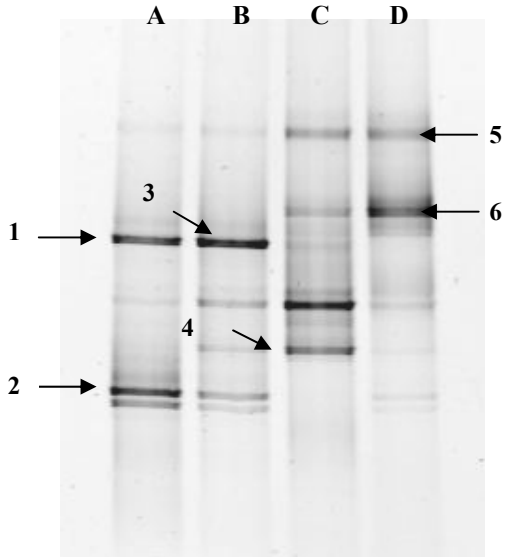

Figure 2 - DGGE-profiles of 16S rRNA gene fragment eubacterial populations at each experimental conditions: (A) 629.8 mg/L, (B) 1183.7 mg/L; (C) 1815.6 mg/L and (D) 4127.6 mg/L.

Table 2 - Affiliation of DGGE fragments determined by 16S rRNA sequence

Band	Affiliation	Accession n° (Genbank)	Similarity (%)	*References*
1	***Enterobacter cloacae***	EF120473.1	98	Feng, R.H.(2006). Not pub.
2	***Burkholderia cepacia***	DQ387437.1	98	[13]
3	***Enterobacter cloacae***	EF059865.1	97	Iversen,C. et al. (2007). Not published.
4	***Clostridium* sp.**	DQ196619.1	90	[14]
5	***Clostridium* sp.**	AY862512.1	98	Zhang, T. (2004). Not published
6	***Clostridium acetobutylicum***	U17030.1	96	[15]

4. Conclusions

The conditions (1) and (2) showed generation of H2 in the early hours of operation showing that the concentrations of substrate were not inhibit to biomass. The conditions (3) and (4) showed lag phase of the generation of H2. The condition (4) had reduced rates of production of H2. The conditions imposed on high concentrations of substrate have become toxic to biomass purified leading slowness in the process of generation of H2.

The best efficiency of sucrose conversion to H2 (1.6 mol H2/mol sucrose) was obtained for the condition (2). The maximum specific H2 production rate (16187.5 H2 µmol/g VSSV.h) was obtained for the condition (3).

Analysis of molecular biology have revealed that organic production of H2 was due to the presence of the species Enterobacter cloacae, Clostridium acetobutyricum and Clostridium sp.; recognized as H2 and volatile acids producers.

Acknowledgements Acknowledgements are due to the Brazilian Research funding institutions Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (process number 04/13085-5)

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Evidence of a bimodal effect on *Saccharomyces cerevisiae* UE-ME_3 by vanadium (V) stress – a dual response to different V_2O_5 medium concentration detected in the rate growth, GSH/GSSG, G6PD, CAT T and GR enzymatic activities

I. Alves-Pereira[1,2], **T. Rosado**[1], and **R. Ferreira** [*,1,2]

[1] Departamento de Química, Universidade de Évora, Apartado 94, 7002-554, Évora, Portugal
[2] Instituto de Ciências Agrárias Mediterrânicas (ICAM), Apartado 94, 7002-774 Évora, Portugal

[*]Corresponding author: Rui Manuel Alves Ferreira, Departamento de Química, Universidade de Évora, Apartado 94, 7002-554 Evora, Portugal, Tel.: +351 266745313; fax: +351 266745394, E-mail address: raf@uevora.pt (R. Ferreira).

The aim of this work was to evaluate the effects of five V2O5 medium concentrations ranging from 0.5 to 2.0 mM on cell viability, redox status and antioxidants enzymes of wine yeast S. cerevisiae UE-ME3. A slightly decrease of yeast cells growth rate for 0.5 and 1.0 mM, and a significantly decrease for 1.5 and 2.0 mM were observed. Conversely, a significantly increase of G6PD activity and GSH/GSSG ratio for 0.5 mM V2O5, and a significantly decrease of GR and CAT T activities for 0.5 and 1.0 mM also occurs. Furthermore, for V2O5, ranging between 1.0 and 2.0 mM, we observed a significantly decrease of G6PD and GSH/GSSG ratio, occurring, at the same conditions a reverse effect on GR and CAT T activities, with a significant increase of GR for 1.5 and 2.0 mM. We suppose that bimodal response of S. cerevisiae to vanadium pentoxide, eventually mediated by NADPH and GSH level, rule cell death.

Keywords *S. cerevisiae*; oxidative stress; vanadium

1. Introduction

Vanadium is a transition metal widely distributed in nature, which is present as trace metal in fossil fuels, whose combustion comprise a significant environmental source of contamination. Despite V is essential for living organisms, including fungi, elevated concentrations of this metal can result in growth inhibition and toxicity responses [1-3]. Consequently, environmental scientists have declared vanadium as a potentially dangerous chemical pollutant. In addition vanadium oxide (V2O5) is described in literature as very toxic compound whose molecular effects are poorly studied. In general, the toxicity of metals can result from reactive oxygen species (ROS) generation that may cause wide-ranging damage in biological molecules and eventually cause cell death [4-9].

Yeast display several antioxidant molecules like GSH and antioxidatives enzymes against ROS, including catalase T (CAT T, EC 1.11.1.6) and glutathione reductase (GR, EC 1.6.4.2), capable of removing oxygen radicals and their products and/or repairing oxidative damage [10, 11]. Glutathione recycling is dependent on the maintenance of an intracellular pool of NADPH, mainly via the pentose phosphate pathway, where glucose-6-phosphate dehydrogenase (G6PD, EC1.1.1.49) is the regulator step [10, 12-14]. Consequently, an adequate intracellular level of NADPH is also required to protect cells against oxidative stress, because GR carries out the enzymatic reduction of glutathione disulfide (GSSG) to glutathione (GSH) at the expense of the reducing power of NADPH. However several authors are also described in Schizosaccharomyces pombe a product inhibition of G6PD by elevated intracellular level of NADPH [15, 16]. In this case the reducing power for glutathione recycling can be eventually generate by isocitrate dehydrogenase (EC 1.1.1.41) [16]. Subsequently, the main purpose of this work was to evaluate the effects of five medium [V2O5] (0.5, 1.0, 1.5 and 2.0 mM) on cell viability, GSH/GSSG ratio, G6PD, GR and CAT T activities of wild wine yeast Saccharomyces cerevisiae UE-ME3.

2. Materials and methods

2.1. Microorganisms and growth conditions

The eukaryotic model used was the wine yeast *Saccharomyces cerevisiae* UE-ME_3, a strain isolated from regional wine (Alentejo-Portugal) belonging to the Enology laboratory collection of Évora University. The

isolated colonies of strain were stored in glycerol (30%, w/v) at -80ºC. The cells were grown to mid-exponential phase in a water bath, with orbital stirring, at 28ºC, in 250-ml flasks containing 100 ml of YEPD medium with 2% (w/v) of glucose. The cells (10^6 cells ml^{-1}) at mid-exponential phase were inoculated in the same condition and incubated during 200 min at 28ºC in the absence or presence of 0.5, 1.0, 1.5 and 2.0 mM V_2O_5. At final time, samples from each treatment were diluted and plated on YEPD medium, in order to obtain viable counts.

2.2. Glutathione and protein determination

Cells growing in YEPD medium with 2% (w/v) of glucose and incubated during 200 min at 28ºC in the absence or presence of 0.5, 1.0, 1.5 and 2.0 mM V_2O_5 were harvested and ultra-sonic disrupt. The resulting extracts were differentially centrifuged at 3000 *g* and 12000 *g*.

The obtained post-peroxisomal supernatant were used for glutathione (GSH) and glutathione disulfide (GSSG) determination according to the spectrofluorometric method of Hissin and Hilf [17] which was based on the reaction of o-phthalaldehyde (OPT) as a fluorescent reagent with GSH in 0.1M sodium phosphate and 0.005M EDTA buffer pH 8.0, and with GSSG in 0.1M NaOH, pH 12.0. GSH was complexed to 0.04M *N*-ethylmaleimide (NEM) to prevent interference of GSH with measurement of GSSG. Fluorescence at 420 nm was determined with the excitation at 350 nm. A series of GSH standards were prepared in phosphate and EDTA buffer, pH 8.0, ranging from 0 to 100 μM and a series of GSSG standards were prepared in 0.1M NaOH, ranging from 0 to 25 nM. The assays were performed using a single beam Shimadzu RF-5001 PC luminescence spectrometer.

Protein concentration was determined according to Lowry [18] using BSA as standard.

2.3. Enzymatic assays

Glucose-6-phosphate dehydrogenase (G6PD) activity determination was based on the increase in absorbance at 340 nm resulting from $NADP^+$ reduction according to Postma [19]. The reaction mixture consisted of: 0.4 mM $NADP^+$(disodium); 5.0 mM $MgCl_2$; 5 mM glucose-6-P and adequate concentration of post-peroxissomal supernatant in 50 mM Tris-HCl buffer pH 8.0.

Glutathione reductase (GR) activity was determined by a NADPH-coupled assay, reading the decrease in absorbance at 340 nm [20]. The reaction mixture consisted of: 0.5 mM EDTA; 2 mM GSSG; 0,15 mM NADPH and adequate concentration of post-peroxissomal supernatant in 100 mM phosphate buffer pH 7.2.

Catalase T (CAT T) activity was determined by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption according to Beers and Sizer [21]. The reaction mixture consisted of: 30 mM H_2O_2 and adequate concentration of post-peroxissomal supernatant in 50 mM phosphate buffer pH 7.5.

Protein and all enzymatic measurements were carried out with a double beam spectrophotometer, Hitachi-U2001.

2.4. Statistical analysis

All the data presented are mean values performed in five independent experiments ± SD. The statistical analysis of results were realized by ANOVA I and Dunett's tests were carried out to determine significant differences ($p<0.01$) between treatments [22].

3. Results

In order to study the effects of V_2O_5 on growth, redox status and enzymatic antioxidant defences of wild-type wine yeast *Saccharomyces cerevisiae* UE-ME_3, cells grown to mid-exponential phase in YEPD medium with 2% (w/v) of glucose were harvest and inoculated in the same conditions and incubated during 200 min at 28ºC in the absence or presence of 0.5 to 2.0 mM V_2O_5. Glutathione contents and enzymatic activities were determined using post-peroxisomal fraction of yeast cells. In each graphic are represented the mean values of five independent experiments.

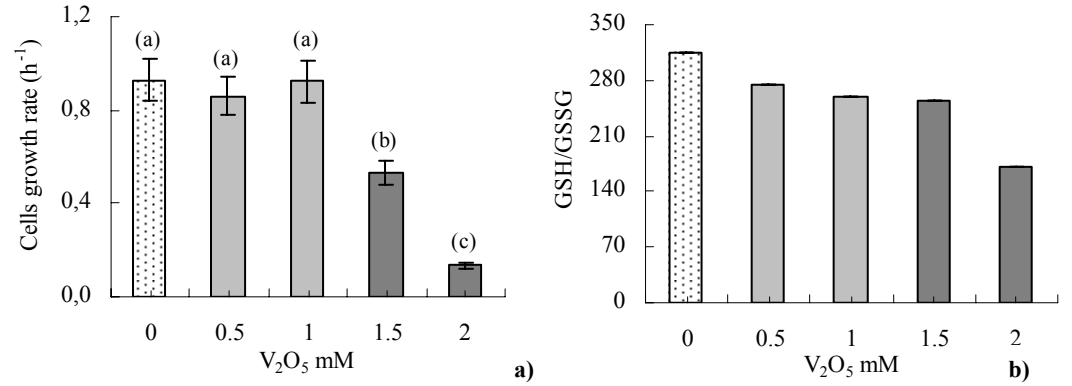

Fig. 1 Effects of vanadium pentoxide on growth and redox status of *Saccharomyces cerevisiae* UE-ME$_3$ growing in YEPD medium with 2% (w/v) of glucose during 200 min at 28ºC in the absence or presence of 0.5, 1.0, 1.5 and 2.0 mM V_2O_5: **a)** Cells growth rate **b)** GSH/GSSG rate. The data shown are means from five independent experiments ± SD. Bars with no common letter are significantly different ($p<0.01$).

Figure 1 shows a bimodal response of *S. cerevisiae* to V_2O_5 concentration ranging from 0.5 to 2.0, were we can observe a slightly effect on yeast cells growth rate from 0 to 1.0 mM, and a significantly decrease (58% and 14% of control) for 1.5 and 2.0 mM, respectively. Identical response can be observed in redox status of the cell, since we can observe a slightly decrease (87% of control) of GSH/GSSG ratio for 0.5 mM V_2O_5 and a significantly decrease (82%, 81% and 54% of control) for 1.0, 1.5 and 2.0 mM V_2O_5.

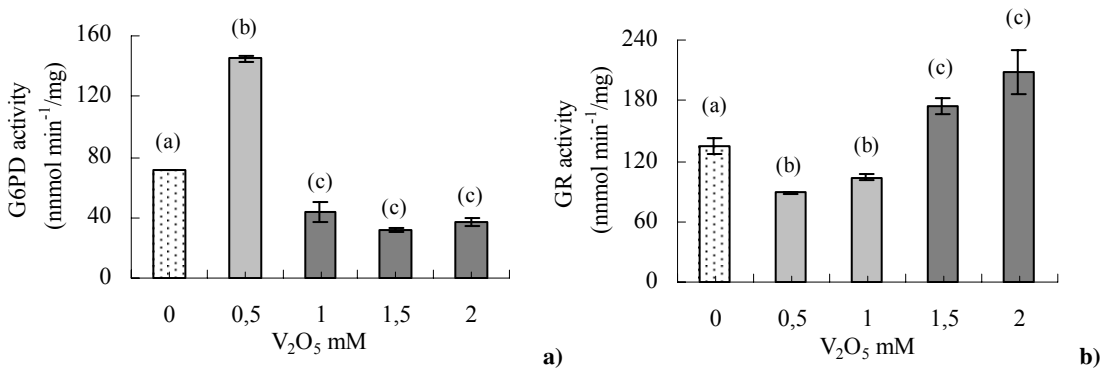

Fig. 2 Effects of vanadium pentoxide on the reducing equivalents production in the form of NADPH for anabolic activities and GSH for detoxication activities of *Saccharomyces cerevisiae* UE-ME$_3$ growing in YEPD medium with 2% (w/v) of glucose during 200 min at 28ºC in the absence or presence of 0.5, 1.0, 1.5 and 2.0 mM V_2O_5: **a)** Glucose-6-phosphate dehydrogenase (G6PD) activity **b)** Glutathione reductase (GR) activity. The data shown are means from five independent experiments ± SD. Bars with no common letter are significantly different ($p<0.01$).

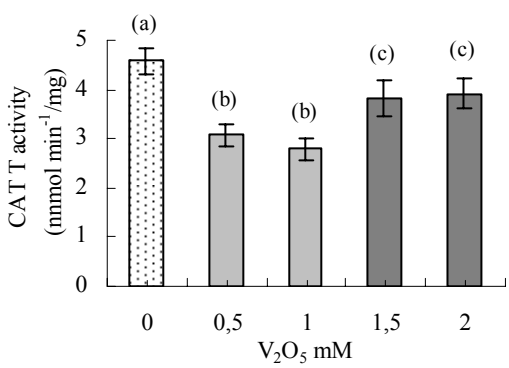

Fig. 3 Effects of vanadium pentoxide on the catalase T (CAT T) activity of Saccharomyces cerevisiae UE-ME3 growing in YEPD medium with 2% (w/v) of glucose during 200 min at 28ºC in the absence or presence of 0.5, 1.0, 1.5 and 2.0 mM V2O5. The data shown are means from five independent experiments ± SD. Bars with no common letter are significantly different ($p<0.01$).

Figure 2 and 3 shows equally a bimodal response at level of antioxidant enzymes. Conversely we can observe a significant increase of G6PD activity (2 fold) for 0.5 mM V_2O_5, and a decrease of GR and CAT T activities for 0.5 and 1.0 mM. Moreover we can observe a decrease of G6PD (50% lower than control) for V_2O_5

concentration ranging between 1.0 and 2.0 mM, occurring, at the same time, a make up of GR and CAT T activity, which in the case of GR the increase is 29 % and 54 % than control, for 1.5 and 2.0 mM, respectively.

4. Discussion

Vanadium is a metal widely used in industry and consequently it is a pollutant in several regions of the planet. Thus, the main purpose of this work was to evaluate the effects of V_2O_5, on cell growth, redox status and enzymatic antioxidant defences of the wild wine yeast *Saccharomyces cerevisiae*, strain UE-ME_3. When reactive oxygen species (ROS) are produced in yeast cells as normal by products of cell metabolism, the molecular defence mechanisms, such as glutathione (GSH) and Catalase T (CAT T), glutathione reductase (GR) and glucose 6-Phosphate dehydrogenase (G6PD) enzymes, under physiological conditions, are able to contribute to avoid oxidative damages [7, 8, 9]. Moreover, this balance could be disturbed when yeast cells are exposed to metal stress conditions, such as vanadium pentoxide. In these work we observed a bimodal response of *S. cerevisiae* UE-M_3 to vanadium pentoxide, in terms of growth rate, GSH/GSSG ratio, and G6PD, GR and CAT T enzymatic activities which is dependent of vanadium medium concentration. Conversely, the results shows that a decrease of cell redox status caused by vanadium pentoxide is significantly correlated (R=0.902) with cell growth rate. On the other hand, the bimodal response of yeast cells at level of antioxidant enzymes, namely inhibition of GR and CAT T when G6PD was activated in cells exposed to 0.5 mM, appoint us to a reductive inactivation mechanism of antioxidants enzymes, which is eventually mediated by intracellular level of NADPH and GSH. Again, the reverse effects which occur from 1.0 to 2.0 mM vanadium pentoxide, like G6PD inhibition, avoid the contribution of pentose phosphate pathway to the synthesis of ribose 5-phosphate, required for the biosynthesis of some amino acids, nucleotides, and coenzymes indispensables to cell growth. In other hand, the GR activation correlated with reverse inhibition of CAT T, for 1.5 and 2.0 mM V_2O_5, can result from an amendment response of these enzymes to the oxidative stress that use intracellular NADPH for reduction of GSSG to GSH, via glutathione cycle, which isn't enough to prevent cell death [12, 15, 16]. These results appoint us that stress conditions generate by vanadium pentoxide rule the cell death.

Acknowledgements This work was supported by "Instituto de Ciências Agrarias Mediterrânicas" (ICAM) at University of Évora, Portugal. We thanks to Engº Paulo Laureano of the Laboratory of Enology of University of Èvora for the yielding of the yeast strain.

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Factorial design applied to biosurfactant production by *Chromobacterium violaceum*

Adriana Almeida Antunes[1,3]; A. M. A. T. Jara[2,3]; J. M. Luna[1,3]; C. D. C. Albuquerque[3]; A. S. Messias[3]; C. A. Alves-Silva[3]; G.M. Campos-Takaki[3,*]

[1]Pós-graduação em Ciências Biológicas, Universidade Federal de Pernambuco, Brazil;
[2]Mestrado em Desenvolvimento de Processos Ambientais/UNICAP, Brazil;
[3]Núcleo de Pesquisas em Ciências Ambientais (NPCIAMB), Universidade Católica de Pernambuco, Recife-PE

[*]Corresponding author takaki@unicap.br galba_takaki@yahoo.com.br

The aim of the present work was to analyze the production of biosurfactants agents from two strains of *Chromobacterium violaceum*, UCP 1467, isolated from Amazons water, and the strain UCP 1489, was isolated from Pernambuco soil, respectively. The condition it was observed with UCP 1467 the reduction of the superficial tension of the water of 72mN/m to 33,24mN/m. whereas the strain UCP 1489 showed lower tension f 31m/Nm. The index of emulsification of the metabolic liquid of free cells was determined for different substratum, 60% of emulsification with canola oil for strain UCP 1467 (condition 6) and in conditions 2, 3, 6 and 11 for strain UCP 1489 getting 31% of emulsification, both with agitation. The activities of emulsification had shown resulted between 4.8 and 6.0 U.A.E. for UCP 1467 whereas UCP 1489 showed to activities of 6 U.A.E. for all tested substratum. The results indicated that strain UCP 1467 for index emulsification and the strain UCP 1489 as good producer of biosurfactant with low superficial tension and high emulsification activity.

Keywords *Chromobacterium violaceum*, biosurfactant, emulsification activity, superficial tension

1. Introduction

Biosurfactants are a wide class of amphipathic molecules that is capable to reduce surface and interfacial tension between gases, liquids and solids with a large industrial variety and environmental applications, as due lower toxicity, biodegradability and effectiveness in a narrow pH and temperature range. However, these limitations, the interest on biosurfactants (surfactants of microbial origin), produced by certain bacteria, yeast and fungi, has increased due to their low toxicity, biodegradable nature, effectiveness at extreme temperature, pH, salinity and their role in saving natural ecosystem by enhancing the biodegradation during hydrocarbon spills [1,2]. The bacteria are the largest responsible for the production of these compounds. These microorganisms have been isolated of the soil, sea water, sediments, and polluted areas. The biosurfactants have several advantages over chemical surfactants including lower toxicity and higher biodegradability, and effectiveness at extreme temperatures or pH values [3,4]. In spite of the advantages, fermentation must be cost competitive with chemical synthesis, and many of the potential applications that have been considered for biosurfactants depend on wither they can be produced economically. Fermentation medium can be representing almost 30% of the cost for a microbial fermentation [5,6,7]. Complex media commonly was employed for growth and lactic acid by bacteria is not economically attractive due to their high amount of expensive nutrients such as yeast extract, peptone and salts [8,9,10].

2. Material and Methods

2.1 Microorganism

The microorganism used was *Chromobacterium violaceum*, UCP 1489 (isolated of small river by Silva (2006) [9] from contaminated area of Pernambuco State, Brazil), and strain UCP 1467, isolated in Amazon, from samples of water and soil respectively and deposited in the Cultures Collection of the Catholic University of Pernambuco, Brazil. Both strains were maintained in Nutrient Agar (Difco) containing (w/v): beef extract (3g/L), Peptone (5g/L), and agar (15g/L) at 5°C.

2.2 Collect and isolation

A water sample was collected in Paca River located in the city of Camaragibe, Pernambuco. The water was collected and kept in bottle amber and submitted to the isolation and identification of microorganisms.

Technique of the multiple pipes was used, being carried through the presumptive assay, using 10 mL of the sample and inoculated in Petri dishes containing nutritive agar, and incubated at 30°C, for 24 hours for counting of the colonies. The microorganisms isolated from contaminated water produced violet colonies, characterized as *Chromobacterium violaceum*. The strain was deposited as UCP 1489 and the first one of the work. The second one, UCP 1467, was isolated in soil sample of the Amazon region [9].

2.3 Media, Cultivations conditions and Biosurfactant production

The strains of *Chromobacterium violaceum,* UCP 1489 and UCP 1467, were transferred from Nutrient agar to Luria Bertani (LB) [10] solid medium Petri dishes [tryptone - (10g/L); yeast extract - (5g/L); NaCl - (5g/L); glucose - (5g/L)] at 30°C for 24-48 hours, then, a loopful of culture was transferred to Erlenmeyer flasks of 500mL capacity containing 100mL of Luria Bertani liquid medium [only tryptone - (10g/L) and yeast extract - (5g/L) as base] added with different concentrations of glycerol (2.5%, 5.0% and 7,5%), sodium chloride (0.5%, 2.75% e 5.0%) and industrial corn (2.5%, 5.0% and 7.5%) in shaker at 150 rpm and stationary condition for 72 hours at 30°C for the biosurfactant production. The pH was adjusted to 7.0 and the Luria Bertani culture contained an initial optical density of 0.5. After 72 hours of growth it was analyzed the superficial tension, the activity and index of emulsification of the strains in both conditions: in shaker and stationary.

2.4 Emulsification activity

Emulsification activity was measured using the method described by Cooper and Goldenberg [11]. We used 6 mL of n-hexadecane, and the oils canola, soy, and babasu oil were added to 4 mL of the culture broth free of cells in a graduated screw cap test tube and vortexed at 3000 rpm for 2 minutes. The emulsion stability was determined after 24 hours, and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.5 Surface activities

Surface tension and critical micellar concentration (CMC) were determined on cell free broth obtained by centrifugation the cultures at 10000 X g for 15 minutes with an automatic Tensiometer (Sigma 70-KSVLTD/Finland) according Kuyukina [12], using the Du Nouy ring method at room temperature. The CMC was determined by measuring the surface tensions of dilutions of cell free broth in distilled water up to a constant value of surface tension.

2.6 Factorial planning

The complete factorial planning of 2^3 with 4 repetitions of a central point was accomplished to analyze the main effects, biomass production and the biosurfactant produced by *Chromobacterium violaceum* (Table 1).

Table 1 - Factorial design of 2^3 and 4 repetitions of a central point applied to biosurfactant production by *Chromobacterium violaceum*.

Conditions	**LB + Glycerol mL/L)**	**LB + NaCl (g/L)**	**LB + CORN STEEP (g/L)**
1.	-1	-1	-1
2.	+1	-1	-1
3.	-1	+1	-1
4.	+1	+1	-1
5.	-1	-1	+1
6.	+1	-1	+1
7.	-1	+1	+1
8.	+1	+1	+1
9.	0	0	0
10.	0	0	0
11.	0	0	0
12.	0	0	0

Level +1: glycerol 7.5(ml/L), sodium chloride 5.0 (g/L), corn steep 7.5 (g/L); Level -1: Glycerol 2.5(ml/L), sodium chloride 0.5(g/L), corn steep 2.5(g/L),; Level 0(%): glycerol 5.0(ml/L), sodium chloride (g/L),2.75 , corn steep 5.0(g/L),.

3. Results and Discussion

The superficial tensions of the *C. violaceum* UCP 1489 reduced of the superficial tension of the water from 72 mN/m to 33 mN/m (condition 5), whereas the strain UCP 1467 showed the lower tension of 31 mN/m (condition 8), both in stationary condition (Table 2). In agreement with results obtained with the same strain, UCP 1489, by Antunes et al., [13] in Luria Bertani broth medium added of different types of oils (soy, canola, corn or babasu) showed significant reductions of the superficial tension, once the tensions founded were around 27 mN/m in shaker. The index of emulsification of the metabolic liquid of free cells was determined for different substratum, 60% of emulsification with canola oil for strain UCP 1467 (condition 6) and in conditions 2, 3, 6 and 11 for strain UCP 1489 getting 31% of emulsification, both with agitation. The activities of emulsification had shown resulted between 4.8 and 6.0 U.A.E. for UCP 1467 whereas UCP 1489 showed activities of 6 U.A.E for all tested substratum. The best results showed UCP 1467 strain the higher emulsifier index, and UCP 1489 as good producer of biosurfactant with lower superficial tension and higher emulsifier activity.

Table 2 - Strains of *Chromobacterium violaceum*, UCP 1467 and UCP 1489 with the superficial tensions of each condition after 72 hours of cultivation in shaker and stationary condition.

Number of condition	UCP 1467 (shaker at 150 rpm)	UCP 1467 (stationary condition)	UCP 1489 (shaker at 150 rpm)	UCP 1489 (stationary condition)
1.	38,57	40,63	38,37	34,33
2.	34,15	36,62	39,68	35,43
3.	40,95	36,27	41,15	40,30
4.	37,90	40,39	40,51	36,58
5.	33,67	38,00	50,10	33,24
6.	34,13	35,86	37,09	33,69
7.	39,83	36,60	41,17	39,63
8.	38,57	31,20	39,41	36,87
9.	34,44	36,02	40,20	37,20
10.	35,49	36,50	42,60	37,71
11.	35,00	37,68	40,34	36,76
12.	35,76	37,04	35,29	37,37

The table 3 showed comparative production of biosurfactant production during 24, 48 and 72h of fermentation using different substrates. The best condition was showed at 24h using soy oil (hydrophobic substrate) followed glucose (soluble substrate).

Table 3 - Biosurfactant production by *Chromobacterium violaceum*, (UCP 1489), using different substrates and the superficial tensions of each condition for fermentation.

C. violaceum UCP 1489	24 HOURS	48 HOURS	72 HOURS
Soy oil	26.00	26.31	27.86
Corn oil	26.16	27.05	27.32
Canola oil	26.58	27.04	27.13
Glucose	26.07	27.15	27.61

In Pareto diagram of the effects standard is illustrated in Figure 1, to a level of 95% confidence, it can be observed that sodium chloride was the factor that, with statistical significance, more favored increasing the surface tension when its concentration became the level -1 for the level +1. The glycerol and corn oil , in that order, were the factors that with statistical significance, more increasing the surface tension, or more favored the reduction of the surface tension, when their concentrations passed to the level -1 for the level +1. The interaction between the glycerol and corn oil also showed statistical significance, and increase surface tension. The interaction of sodium chloride and the corn oil favored, not significantly, the increase surface tension, and the interaction of glycerol with sodium chloride do not favored, although not significantly, increased surface tension, or not significantly, the reduction of the surface tension.

In Pareto diagram, Figure 2 showed the effects to a level of 95% confidence, it can be observed that the factors, corn steep, glycerol and sodium chloride, in that order, do not favored, with statistical significance reduction of the surface tension, when their concentrations passed the level -1 for the level +1. The interaction between the glycerol and corn steep do not favored, with statistical significance helped significantly to reduce the surface tension. The interaction of sodium chloride with corn steep do not favored, not significantly helped, but does not significantly, the reduction of the surface tension. And, the interaction of glycerol with sodium chloride favored, not significantly the reduction of the surface tension.

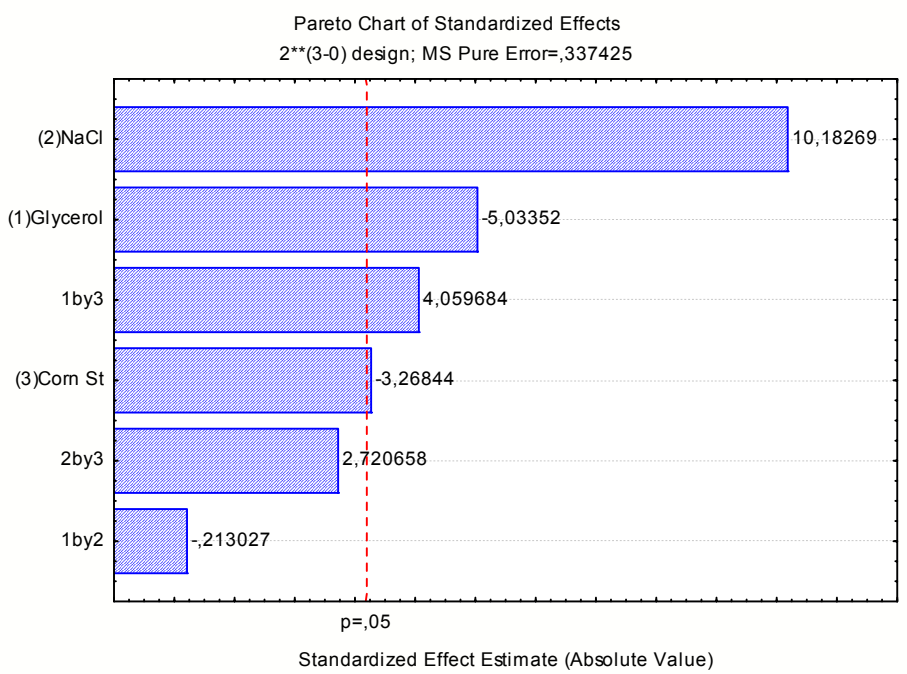

Figure 1 – Pareto Diagram of the effects standards. The independent variables: glycerol, sodium chloride, corn steep, and corn oil, and the dependent variables: superficial tension. Microorganism: UCP 1467, and condition of incubation: 150 rpm

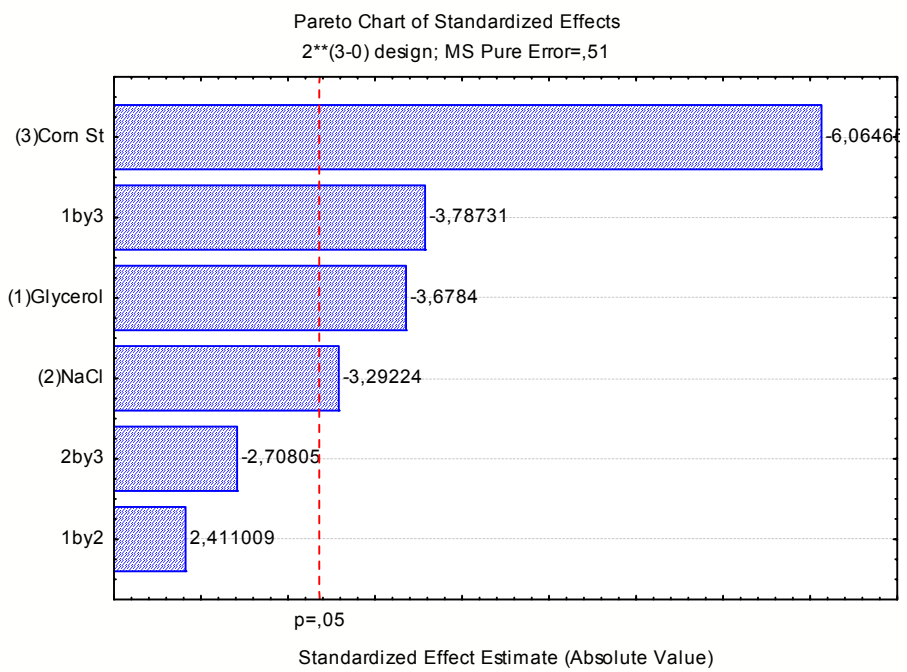

Figure 2 – Pareto Diagram of the effects standard. Independent Variables: glycerol, sodium chloride, corn steep. Dependent variable: surface tension. Microorganism: UCP 1467. Condition of incubation: static

4. Conclusions

The best results indicated that strain UCP 1489 as good biosurfactant producer with lower superficial tension and higher emulsifier activity index. In conclusion the results demonstrated the potential of the *C. violaceum* for the biosurfactant production, and indicating future perspectives for bioremediation process.

Acknowledgments The authors are grateful to CAPES, FACEPE, CNPq and FINEP

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Formation of Biofilms and Production of Enzymes by *Bacillus subtilis* on Surfaces of Polyethylene Terephtalate Simulating Degradation

M. A.T. Jara[1], A. A. Antunes[2], N. S. E. Barreto[3], R.D. Rufino[4], N. E. Duran, C.D.C. Albuquerque[5], A. E. Nascimento[5] and G. M. Campos - Takaki[5]

[1] Master degree in Development of Environmental Processes, Catholic University of Pernambuco – UNICAP, Recife-PE
[2] Doctorate in Biological Sciences, UFPE/Recife-PE
[3] Institute of Sciences of the Sea-Federal LABOMAR, Universidade of Ceará, Fortaleza, CE, Brazil
[4] Doctorate degree in Fungal Biology, Federal University of Pernambuco, Recife-PE, 50.670-420, Brazil
[5] Nucleus of Reserche in Environmental Sciences (NPCIAMB), Catholic University of Pernambuco, 50.050-590 Recife-PE, Brazil
[6] Chemistry Institute, UNICAMP-São Paulo and UMGC-São Paulo, São Paulo, Brazil

The modification of polyethylene terephthalate (PET) fibres from used beverage bottles was investigated by treatment with UV (6 and 36h), temperature (35ºC and 50ºC), and without physic treatment on the production of extracellular enzymes, and biofilm formation by *Bacillus subtilis* under controlled conditions. The results showed partial degradation of the copolymer submitted to physic treatments and colonization by *B. subtilis*. The best results of degradation were associated with protease, amylase and esterase on surface of PET particles submitted to 50^0C of temperature, during 60 days. However, the esterase activity simulating biodegradation of PET by *B. subtilis*, and suggest residual lost of weight, and the products showed low toxicity when compared with the PET particles without treatments.

Keywords polyethylene terephtalate; ezymes and *Bacillus subtilis*

1. Introduction

In recent years, the man comes discovering the necessity of the preservation of the environment where he lives. The synthetic polymer use has significantly increased since the start of century XX [1]. In accordance with estimates, about 100 million tons of these materials are, currently, produced in the world, per year [2]. Practically, a half of all this production is discarded quickly, remaining in deposits of garbage and sanitary landfills per decades. In this way, the synthetic plastics come if accumulating in the nature, about 25 million tons/year [3]. Plastics materials are formed by the union of great called molecular polymer chains, which in turn, are formed by lesser molecules, monomers. The plastics are produced through a chemical process, polymerization that provides the chemical monomer union to form polymers [4]. According to [5] the PET was introduced in the Brazilian market since 1988, and is one of used polymers more in the present time mainly in the manufacture of packing's for cooling. The PET is persistent synthetic polymers in the environment do not have comments to be incinerates in municipal garbage for generating the gas chloride of hydrogen, potentially generating toxic substance and of acid rains, where nor always the incineration is viable for the final treatment of the discarted material [6]. The plastic recycling has been used in some cases. The biodegradation of polymers is a process in which filamentous bacteria, fungus and leavenings express its enzymes to consume part of the chemical structure, as carbon source, modifying of original form the polymer until its complete degradation as food modifying the original form of the material until its disappearance in the environment [7].

The existence of microorganism's capable to degrade xenobiotics composites is of great interest for the bioremediations [8]. It enters, the bacteria *Streptomyces virisosporus* and fungus *Phanerochaete chrysosporium*, are known as degradate of lignin [9]. In this work was investigated the ability of *Bacillus subtilis* in the biofilm formation on the surface of polyethylene terephtalate, using previous treatment of the temperature (35ºC and 50ºC) and the ultraviolet ray irradiation (6 and 36 hours) during 72h, evaluating the enzymes productions (polyphenoloxidase, amylase, protease and esterase), and toxicities of the products formed by degradation process.

2. Materials and methods

2.1 Microorganism

The *Bacillus subtilis* UCP 999 was obtained from the culture collection of the Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Brazil, and maintained in nutrient agar medium at 5ºC.

2.2 Polyethylene Terephthalate-PET

In the experiments were used pieces of 20 x20 mm contained 0.35 mm of tight were cut from PET bottle. The PET was submitted the following treatments: a) UV irradiation during 6 and 12 hours, and 40 cm of distance of the UV lamp (2.400 and 2.800 nm.); b) the effect of the temperatures of 35ºC and 50ºC, and was carried out during 72 hours, respectively. Controls were done without treatments. The pieces previously had been cut and weighed and placed for disinfection process with iodized alcohol during 60 minutes, and after this time washed with distilled water, and was treated twice with ethanol pure per more 60 minutes, and after removed dried according to ASTM-D-5247, [11]. At final, washed with water per more 60 minutes and the plastics had been leaved to the ambient temperature for drying. All the disinfection procedure was done for all pieces of PET treated and control, and all procedures' were made inside of the laminar flow.

2.3 Biofilm formation

The pre-inoculums was carried out using Erlenmeyer's flasks of 125 mL, containing 25 mL of nutrient broth medium, following inoculation with *Bacillus subtilis*, maintained in orbital shaker (150 rpm), at 35ºC, during overnight. The pre-inoculums ($O.D_{600nm}$= 0.5) were transferred to flasks of 250 mL, of capacity containing 50 mL of nutrient broth medium, added 0.05% of tween 80 before sterilization [12], and added of the PET pieces (control and treated), incubated in orbital shaker at150 rpm, at 35ºC , during 30 and 60 days. The incubation period, the cultures were removed and evaluated the viability. The cellular viability was carried out by pour plate technique using Petri dishes and nutrient agar, incubated during 24h, at 35ºC, and counted the number of viables colonies per mL, UFC/mL. Then the metabolic liquid was filtered with filtter (Sterifil D-GV, Millipore of 0,22μm), and was done the enzymatic detection, pH, total protein, and toxicity.

Two pieces of PET were evaluated the films formation on surface. The biofilm was removed by washes treatment with dodecyl sodium sulfate (2%) during 4h, at room temperature (28^0C), following 5 washes with distilled water, and placed at 35º C until completely dried. Then were transferred to dissector and maintained until constant weight. Controls were performed with the absence of physical treatment. All experiments were performed in duplicate.

Determination of pH: The pH of the metabolic liquid free of cells was determined using pHmetro Orion, model 310.

Determination of total proteins – The total proteins was determined using the metabolic liquid free of cells according to kit LABTEST Diagnostica, at 545 nm, using serum albumin as standard.

2.4 Toxicity evaluation

The toxicity tests were carried out with the metabolic liquid free of cells corresponding to 30 and 60 days of incubation of PET (treated or not), using the crustacean Artemia saline as bioindicator. The bioassay was based only on the percentage of death of the bodies in relation to its total number (10 larvae) in the presence of different concentrations of metabolic liquid (v / v 25%, 50% and 75%), diluted in 5 ml an aqueous solution of synthetic sea salt (33.3 g L), incubated for 24 hours, with maximum volume of the sample tested, 1.5 ml [13]. The counting of bodies survivors, it was determined the dose limit (LC50) of the metabolic liquid. The CL50 of the strains was calculated by Trimmed Spearman-Karber's method [14]. The tests were performed in duplicate

2.5 Detection of Enzymatic Activity

Esterase: The detection of esterase activity was performed by the method described by [15], and Tween 80 as substrate. Disks of paper syrup 6 mm were embedded with 100 μ l, deposited in the center of the plate, Petri containing the means to esterase activity, incubated at a temperature of 35 ° C, being observed every 24 hours, after the appearance of the purple halo around the disc, measured and expressed in mm.

Amilase: The detection of the amylase activity was performed in accordance with the methodology of [16], and soluble starch as substrate. Records of 6 mm role syrup, containing 100 μ l of the liquid metabolic (control and

treated), deposited in the center of the plate, Petri containing the means to amylase, incubated at a temperature of 37 ° C and is observed with 24-emergence of the transparent halo around the disk, measured and expressed in mm after staining with iodine solution.
Protease: The detection of protease activity of the metabolic liquid has been carried out according to the methodology [16], using gelatin to 2% as substrate, being observed by the appearance of transparent halo around the disk, measured and expressed in mm.
Polyphenoloxidases: The determination of the activity of polyphenoloxidases was done using and gallic acid as substrate, being observed by the appearance of the brown halo around the disk, measured and expressed in mm[17].

2.6 Scanning Electron Microscopy

The biofilm formed by *B. subtilis* with 30 and 60 days of the PET particles were washed in PBS, pH 7.2, twice, for 10 minutes, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hour, room temperature. After the stage-setting, all samples were washed with phosphate buffer twice, for 10 minutes, followed by post-fixation with tetroxide at 1% in phosphate buffer, for 1 hour at room temperature, in the absence of light. Then, the samples were again washed with 0.1 M phosphate buffer, and subsequently, subject to the process of dehydration. For the dehydration of the samples was used ethyl alcohol, in proportions of 50%, 70%, 90% (5 minutes for each exchange) by the ratio of 100% (three times, 10 minutes each exchange). After this step, the samples were submitted to the critical point for total elimination of the liquid phase and mounting brackets in aluminum, with further metallization. The samples were observed, analyzed and photographed in the Electronic Scanning Microscope JEOL JSM T2000 [18].

3. Results and Discussion

The table 1 showed the values of cellular viability of *B. subtilis* after 30 days and 60 days of incubation with the particles of the polymer, with and without treatments. It was observed an increase in the number of cells (9.1 $x10^7$) for particles of PET treated with UV irradiation during 6 hours, in relation to the initial number of cells of control. Those results with the growth of *B. subtilis* during 30 and 60 days of incubation shown that the initial pH was 6.72, and increasing (pH> 8.0), in all treatments, including the control. According the literature [19 e 20] , changes in the electrical properties of the plastics particles are due, mainly, to microbial growth on the surface, being associated with the humidity and the changes in pH, probably caused by chemical alteration of the PET constituents, and probably formed secondary metabolites. With respect to total proteins produced in metabolic liquid with 30 and 60 days of incubation, demonstrating that the growth (60 days) is too longer, except for irradiation with UV light 6 hours and the temperature of 50 ° C.

Those results suggest that content of protein in the process of colonization may be associated to enzyme degradation of PET, and that information is corroborated by the results obtained with *Rhodococcus rubber* during the degradation of polyethylene terephtalate [21] .

The particles treated with UV irradiation during 6 hours and temperatures of 50°C, respectively, showed a decrease of 0.06% and 0.07% of the masses of the particle of PET. In this sense, the literature refers to a loss of mass in the percentage of elongation of the polymer (polyclean), but also in the average molecular weight, when compared to the control (no treatment) , when was treated with UV lights[19]. On the other hand, those results are confirmed with the literature which described the biodegradability of polymer depends on some physical and chemistry properties, including the molar increase of mass and ramifications in the chains, which make it difficult biodegradation [22,23].

Table 1: Growth of *Bacillus subtilis*, biofilm formation, total proteins and pH with 30ande 60 days of incubation of the polyethylene terephtalate -PET with and without treatment

PET	Growth of B. subtilis (UFC mL)		Biofilme (mg)		Total Proteins (mg mL)		pH	
	30d	60d	30d	60d	30d	60d	30d	60d
Controle	$3.8x10^7$	$8.4x10^7$	0.06	0.03	32	46	8.40	8.57
UV 6h	9.2x10	$9.1x10^7$	0.12	0.07	42	28	8.11	8.09
UV 36h	$7.4x10^7$	$9.1x10^7$	0.03	0.03	14	52	8.41	8.60
T 35 ºC	$5.7x10^7$	$4.3x10^7$	0.05	0.08	12	28	8.51	8.05
T 50 ºC	$5.3x10^7$	$7.6x10^7$	0.05	0.04	16	8	8.02	8.35

*pH initial 6,72 * Pre-inoculum's= $5,9x10^2$
*Control : particles of PET without traetaments

3.2 Enzymes produced by *Bacillus subtilis*

The growth of *Bacillus subtilis* in nutrient broth added of tween 80 and PET particles were observed the enzyme production as protease, amylase, and esterase, except polyphenoloxidases. The amylase activity produced by *B. subtilis* shown halos of 10 to 48 mm, however, the control demonstrated the highest detection with 60 days of incubation. It was also observed that the amylase produced by *B. subtilis* containing the PET particles treated with UV and temperature the production was more expressive (halos of 40 to 45 mm) with 60 days of incubation. Similar results were also observed with protease, and the control, halos measured 45 mm, with 60 days of incubation. It is emphasized that the treatment with a temperature of 50°C formed the highest halo with 60 days of incubation. The results also demonstrated that only when the polymer was previously treated with a temperature of 50°C was produced esterase (40 mm). The literature refers to the hydrolytic enzymes synthesized by microorganisms such as amylase, protease and mainly, esterase [24], was involved with the process of PET degradation, whose information supporting the results obtained, suggesting a possible degradation of PET in the conditions studied. But, we do not found the production of polyphenoloxidases by *B. subtilis* in all treatments. However, the literature confirmed the polyphenoloxidases production by thermophilic *Bacillus spp*. [25]. Those results may be explain because we was used a mesophilic strain of *B. subtilis*.

3.3 Biofilm formation by *Bacillus subtilis* on surface of PET

The treatment physical-chemistry induced to the plastic material to lose some physical properties and may be become more accessible to the microbial colonization, and consequently, to make possible the biodegradation process [26, 27]. The biodegradation process can be facilitated by previous application of light (UV) and/or heat exposition, and produced alterations into the chemical structure [28].

Our results with both treatments UV irradiation (6h) and temperature (35ºC) demonstrated to be more appropriate for the surface colonization and biofilm formation by *B. subtilis*, when were compared with the particles control (without treatment) (Figure1). A homogeneous and scattered distribution was observed suggesting connection of the biofilm formation by microorganisms on the surface of the polymer (Figure A-C). However, the treatment with temperature of 35ºC was observed high electronic density, the cellular structure is well defined, and those information are corroborated with the process of colonization of *Rhodococcus rubber*[21].

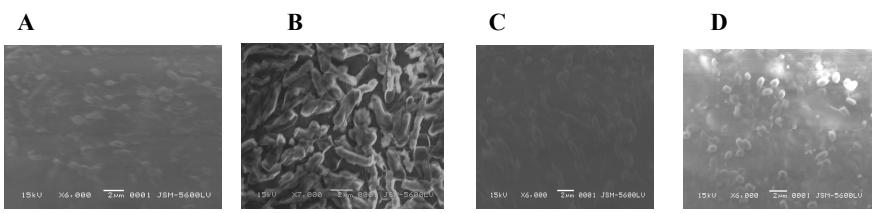

Figure 1. Eletronmicrograph obtained by scanning electron microscope –SEM showing the biofilm formed by *Bacillus subtilis* in the particles of PET, after 60 days of incubation in terephtalate of polyethylene-PET. A - Control (without treatment) 6.000X; B- Treatment with temperature of 35ºC 7.000X; C - Controls (without treatment)6.000X; D: Treatment with 6 hours of UV irradiation 6.000X

3.4 Toxicity of the metabolic liquid

The metabolic liquid obtained after the period of 30 and 60 days of incubation of *B. subtilis* with treatment of UV irradiation (6 and 36 h) was tested for toxicity using *Artemia saline* for analyzed. The controls do not showed toxicity. However, the liquid metabolic with 30 days of incubation showed higher toxicity, and then was compared with 60 days of incubation. However, the irradiation during 36h demonstrated larger toxicity, mortality between 10 to 55%, and consequently, the CL_{50} was impossible to calculate. Other wise, 6h of irradiation showed CL_{50} of 62.24%. The treatment of temperature of 35ºC was observed CL_{50}54.33%, and variance of 43.83% to 67.65%. However, effect of 50ºC of temperature showed more high CL_{50} of 45.74% , and the variance test of the 37.85 to 55.27%, for an interval of confidance of 95%, consequently, we have had higher mortality of *A. saline.* Those studies of toxicity tests were validity by the literature using *A. saline* suggesting the presence of toxic metabolites were produced with UV and temperature treatments [29]. And the significant results of mortality were obtained with UV (36h) and temperature (50ºC).

4. Conclusions

The previous treatment with UV and temperature suggested chemical alterations in the properties of the plastic, which provided conditions for biofilm formation on the surface of the PET by *B. subtilis*. However, the best

treatment utilized in this study was temperature (50ºC), provided a major alteration in the surface of PET, consequently, fast adhesion and colonization of the *B. subtilis*, expression the enzyme esterase, and may be responsible for the degradation phenomenon, loss the polymeric mass, and lower toxicity.

Acknowledgements The authors are grateful to the Brazilian Agencies financial support by CAPES, CNPq and FINEP.

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Glutamylcysteine ligase gene of the ciliated protozoan *Tetrahymena thermophila*: A potential tool for pollution monitoring

R. Ortega, A. Martín-González, and Juan C. Gutiérrez*

Departamento de Microbiología-III, Facultad de Biología, C/. José Antonio Novais, 2. Universidad Complutense (UCM). 28040 Madrid. Spain

*Corresponding author: e-mail: juancar@bio.ucm.es Phone: +34 913944968 Fax: +34 913944964

We have cloned, sequenced and characterized the gene encoding the enzyme glutamylcysteine ligase (GCL), the rate limiting enzyme of the glutathione biosynthesis, in the ciliated protozoa *Tetrahymena thermophila*, a eukaryotic microorganism model commonly used in ecotoxicological bioassays. The GCL gene has 1296 bp and contains an intron. The inferred protein sequence (405 aa) showed a similarity of 21% to GCLs from mammals, a 15-21 % to fungi / yeast GCLs, and 7% to plant GCLs. By qRT-PCR, we have also analyzed the GCL gene expression level after 2 or 24h exposure to different toxic agents and environmental stress conditions. Remarkable overexpression of GCL gene is detected under the presence of non-essential metal/metalloids Cd or As, the oxidants menadione, the herbicide paraquat, as well as under the organic compound CDNB, a well-known substrate of glutathione-S-transferase enzymes. Likewise, an acid environment (pH 5) also induced GCL gene transcription. Several possible roles of glutathione in the cellular resistance to these stress conditions are discussed.

Keywords glutamylcysteine ligase; gene expression; ciliated protozoa; *Tetrahymena thermophila*; biomonitoring pollution.

1. Introduction

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinylglycine), which represents the most abundant cellular non-protein thiol in almost all eukaryotic groups, excepting parasitic protozoa lacking mitochondria, and in many Gram-negative and some Gram-positive bacteria. This molecule has multiple physiological roles in biological systems: scavenger of reactive oxygen species, detoxification of toxic agents, amino acid import, protein and DNA synthesis and cellular redox buffer [1]. This tripeptide is synthesized by two dependent sequential reactions; ligation of L-glutamate and L-cysteine by the catalytic action of the glutamylcysteine ligase (glutamylcysteine synthetase), and then the addition of glycine by the glutathione synthetase enzyme. The first step of glutathione biosynthesis is crucial because it is the primary rate-limiting of the pathway, and the dipeptide can assume, in the absence of glutathione, some roles of this molecule, e.g. as antioxidant [2]. In mammals and insects (*Drosophila*) the enzyme glutamylcysteine ligase (GCL) is heterodimeric; with a catalytic subunit and a regulatory subunit. By the contrary, in plants, nematodes (*Caenorhabditis*), yeasts, parasitic protozoa and bacteria is monomeric [3,4]. During the last years, we have studied the macronuclear genes encoding diverse enzymes involved in the GSH metabolism of the free-living ciliated protozoa *Tetrahymena thermophila*. In this study, for first time, we have cloned, sequenced and characterized the glutamylcysteine ligase gene from this eukaryotic microorganism. Besides, we have analyzed its gene expression level in presence of several toxic agents as heavy metals (Cd, As, Cu or Zn) and oxidants (paraquat (PQ), menadione (MD), or under other environmental stress conditions (like acid/basic pHs or starvation) and the organic chemical CDNB (1-chloro-2,4 dinitrobenzene) (the experimental inducer of the glutathione-S-transferase enzymes).

2. Materials and Methods

We have used the strain SB1969 of *Tetrahymena thermophila*, that was grown axenically in PP210 medium as previously described [5]. Before RNA isolation, exponential cell cultures were exposed to different stress conditions; during 2h (MD, CDNB or heavy metals), 24h (extreme pHs or heavy metals) or 48h (starvation). The used concentrations for each toxic agent were the followings: 2 μg /ml Cd, 20 μg /ml Cu, 7.5 μg /ml As, 250 μg /ml Zn, 200 μg /ml PQ (paraquat), 5 μM MD (menadione) and 20 μM CDNB (1-chloro-2,4 dinitrobenzene). pH treatments were 5.0 for acid conditions and 9.0 for basic environment. All above exposures were carried out in PP210 medium. Starvation was in 0.01 M HCl-Tris buffer (pH 6.8). Exponential 100 ml cultures of *T. thermophila* were harvested by centrifugation at 2.200xg for 2 min. Total DNA was isolated as described [6]. Total RNA was isolated using RNAqueos ™ 4PCR kit (Ambion). All samples were treated with RNase-free DNase I (Ambion), according to the protocol supplied by the manufacturer. GCL gene was amplified by PCR, using primers previously designed from the nucleotide sequence obtained from the complete already sequenced macronuclear genome of this ciliate. Amplified product was cloned using the TOPO TA Cloning kit (Invitrogen). The DNA sequence was determined using an ABI PRISM ™ 377 DNA automatic sequencer (PE Applied Biosystems), according to the dideoxy technique and using appropriate primers (Big-dye™ terminator cycle sequencing ready reaction kit from AP Biosystems). Homology searches were performed using BLAST program at the NCBI website (http://www.ncbi.nlmnih.gov/BLAST/). Multiple sequence alignments were carried out with Align X, using the software program Vector NTI™ Suite 9 (InforMax). Phylogenetic tree of GCL amino acid sequences was constructed by using the UPGMA algorithm (CLC Combined Workbench).

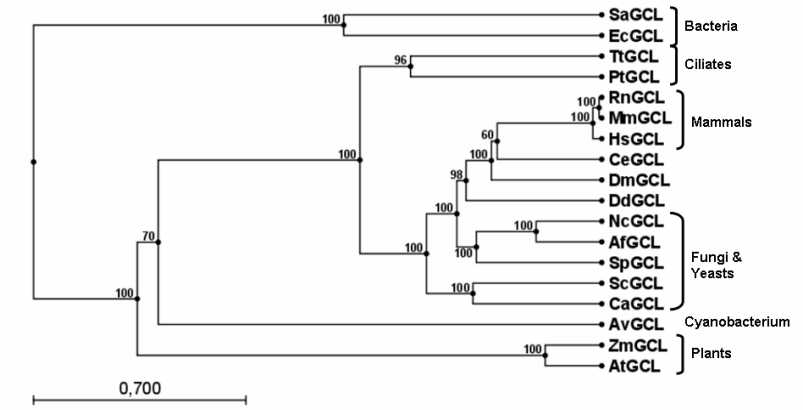
The cDNA synthesis was carried out using 5μg RNA, oligo-d(T) primer (5 μM), AMV reverse transcriptase (Roche) (75 U/ μg RNA) with Rnase inhibitor (25 U) (Roche) and 2.5 mM dNTPs, in a total volume of 20 μl. cDNA samples were amplified in duplicate microtiter plates (Applied Biosystems). Each PCR reaction (20 μl total volume) contained: 10 μl of SYBR Green PCR Master Mix (Applied Biosystems), 1 μl of each primer (at 300 nM final concentration), 3 μl of H_2O and 5 μl of cDNA. The α-tubulin gene was used as an endogenous control gene or constitutive cDNA. Real-time PCR reactions were carried out in an ABI PRISM 7700 real time PCR apparatus. All controls were negative (no template control and RT minus control). To calculate the relative change in expression we used the ΔΔCt method. Real-time PCR reactions were carried out in an ABI PRISM 7700 real time PCR apparatus.

3. Results and Discussion

A new putative gene encoding glutamylcysteine ligase (GCL) was found by BLAST searching in the already published macronuclear genome of the ciliate *T. thermophila*. Appropriate primers were designed and, after standard PCR, the complete ORF was cloned and sequenced. The complete gene has 1296 bp of length and contains a 79 bp intron, located at between +48 and +126. The 1218 bp coding region of the putative GCL gene encodes a predicted protein of 405 amino acids with a calculated molecular weight of about 47.6 KDa. Therefore, in this ciliate, GCL looks like a monomeric enzyme as it occurs in bacteria, parasitic protozoa, fungi and plants (plastids), but unlike invertebrate and vertebrate animals, where GCLs are heterodimers, composed of both catalytic and regulatory subunits [3,4,7].

It must be denoted the exceptional short length of *Tetrahymena* GCL sequence, it is extremely short if we compare with those from other organisms and microorganisms, including fungi (661-728 aa), parasitic protozoa (795-1161 aa), invertebrates (601-717 aa), mammals (601-637 aa) and even certain gamma-proteobacteria or alpha-proteobacteria. Likewise, we have found, in the genome database of *Paramecium tetraurelia* (another free-living ciliate model), a putative GCL sequence of 677 amino acids, which is into the length range of the majority of GCLs. Therefore, amino acid sequence length (405 aa) of *T. thermophila* GCL is unique among eukaryotic microorganisms, being quite similar to that from chloroplasts of certain plants as *Oryza sativa* (438 aa), human pathogenic bacteria (*Mycobacterium tuberculosis* with 432 aa), or that from the cyanobacteria *Anabaena* (379 aa), the shortest reported till now GCL protein [4].

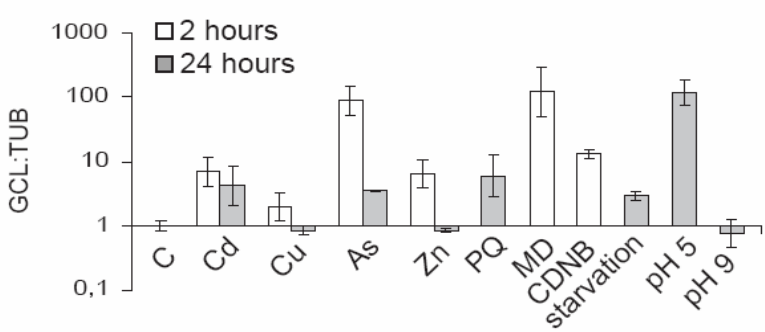
The highest similarity of *T. thermophila* GCL is to those from other free-living protozoa, as *Paramecium tetraurelia* (30.2%) and *Dictyostelum discoideum* (21.7%). Then, the higher similarity (about 21%) is to mammalian (including humans). The phylogenetic tree (Fig. 1) derived from the GCL amino acidic sequence alignments of very diverse microorganisms (prokaryotic and eukaryotic) and different groups of pluricellular organisms shows that ciliate GCLs have a common origin (or common ancestral GCL) with mammalians, invertebrate, fungi and yeast GCLs (all eukaryotic organisms), and all of them are related with cyanobacteria GCLs.



AfGCL (*Aspergilus fumigatus*), AtGCL (*Arabidopsis thaliana*). AvGCL (*Anabaena variabilis*), CaGCL (*Candida albicans*), CeGCL (*Caenorhabditis elegans*), DdGCL (*Dictyostelium discoideum*), DmGCL (*Drosophila melanogaster*), EcGCL (*Escherichia coli*), HsGCL (*Homo sapiens*), MmGCL (*Mus musculus*), NcGCL (*Neurospora crassa*), PtGCL (*Paramecium tetraurelia*), RnGCL (*Rattus norvegicus*), SaGCL (*Streptococcus agalactiae*), SpGCL (*Schizosaccharomyces pombe*), TtGCL (*Tetrahymena thermophila*), ZmGCL (*Zea mays*), ScGCL (*Saccharomyces cerevisiae*).

Figure 1 Phylogenetic tree of GCL amino acid sequences from diverse organisms, using the UPGMA algorithm.

A relevant aspect is the analysis of expression of GCL gene of *T. thermophila*, under diverse pollutants or toxic agents, and other environmental stress conditions. Real time quantitative RT-PCR results are showed in Fig. 2. The highest gene expression inductions are observed when the cellular populations are exposed to Menadione (MD) (an average value of 123 folds induction), or to an acid pH (5.0) (120 folds induction) (Fig. 2). MD (or Vitamin K3) is an oxidant or electrophile (2-methyl-1,4 naphtoquinone) that leads to formation of reactive oxygen species (ROS), such as superoxide, peroxide and the highly toxic hydroxyl radicals [8]. According to diverse authors [9], it is expected that the 3-position free -OH radical of MD reacts with the sulphydryl group of the glutathione by a Michael-type addition. The role of glutathione in acid stress is less known, however, it has been reported in diverse bacteria that glutathione is involved in acid tolerance since it protects to the prokaryotic microorganisms against acid stress [10,11]. Paraquat (PQ) (1-1'- dimethyl 4,4'-bipyridinium ion) is a quaternary nitrogen herbicide that is worldwide used in both terrestrial and aquatic environments. However, it is a highly toxic compound for humans and animals due to its capacity to generate superoxide anions, which can lead the formation of the most toxic ROS, like hydrogen peroxide and hydroxyl radical [12]. Like in yeasts [13, 14], PQ (after 24h treatment) also induced certain over-expression (6 folds induction) (Fig. 2) of the *Tetrahymena* GCL gene, what may be related with the capacity of glutathione to protects cells against the damage caused by oxidants.



GCL:TUB : Fold-induction of GCL expression normalized against the α-tubulin gene. C: Control; PQ: Paraquat; CDNB: 1-chloro-2,4 dinitrobenzene.

Figure 2 Relative expression levels of *T. thermophila* GCL gene obtained by real qRT-PCR, after diverse stress treatments.

CDNB (1-chloro-2,4 dinitrobenzene) is a toxic xenobiotic known to cause oxidative stress and cell death. Glutathione-S-transferases (GSTs) are an almost universal family of enzymes which conjugates CDNB, and other toxicants, mainly organic pollutants, with GSH, to form 2,4-dinitrophenyl-S-glutathione (DNP-SG). This toxic agent is considered as an experimental inducer of GSTs in biological system or even, it may be a positive control of GSH biosynthesis. In *T. thermophila*, the treatment with this compound (CDNB) increased an average of 13 folds the basal expression of the GCL gene (Fig. 2).

Special mention should be made about the acute short (2h) or extensive (24h) treatments with non essential (Cd, As) or essential (Cu, Zn) heavy metals/metalloids. As it was expected, non essential metals caused a high induction of GCL gene expression. This transcriptional induction is higher under short acute exposures (2h) than under more extensive (24h) metal treatments (Fig. 2). After 24h exposure, both metals (Cd or As) raised similar gene expression levels (4 folds induction), despite the chemical nature of these metals is rather different. However, the over-expression of GCL gene is remarkably more intense after short (2h) exposures to As (89 folds) or to Cd (7 folds). Cadmium itself is unable to form free radicals directly, but an indirect generation of various radicals, involving superoxide radical, hydroxyl radical and nitric oxide formation, has been postulated. Besides, the generation of hydrogen peroxide which itself in turn may be a significant source of radicals via Fenton chemistry is confirmed [13]. Both, GCL gene over-expression and an increase of the GCL cell concentration have been detected in cyanobacteria, yeasts and plants [4, 14, 15]. At least, in photosynthetic organisms and yeasts, glutathione biosynthesis should be also related with the phytochelatins formation [16]. In *T. thermophila*, we have stated that Cd induced the synthesis of specific Cd-metallothioneins. [5], but we have not detected the existence of phytochelatins related with Cd bioaccumulation (unpublished data). According to the results, GSH might play directly an important role in cellular resistance to Cd in this ciliate, like it occurs in animals. Many studies have confirmed the generation of free radicals by arsenic cellular metabolism. ROS generation by As is a complex process, which includes the formation of superoxide, singlet oxygen, peroxyl, nitric oxide, hydrogen peroxide, dimethylarsinic peroxyl and dimethylarsinic radicals [13]. The existence of metallothioneins (MTs) and/or phytochelatins involved in As bioaccumulation is not proven in animals, although some evidences indicated that certain mammalian metallothioneins can quelate, at least *in vitro*, cations of this metalloid. *T. thermophila* CdMT genes are induced under As treatment [5]. By other hand, glutathione depletion with L-buthionine sulfoximide (BSO) caused sensitized cells to both arsenite and arsenate [17]. In animals, it has been reported a novel pathway of inorganic arsenic detoxification which involved the GSH conjugation of AsIII to form arsenite triglutathione and subsequent methylation to yield monomethylarsonic, followed by dimethylarcinic glutathione [18]. The enzyme catalyzing arsenic methylation requires reducing agents such as GSH and cysteine. This finding explains the necessity to increase the GSH biosynthesis. In microalgae and plants, in which metallothioneins and phytochelatins are clearly involved in arsenic resistance, three main roles have been attributed to GSH in relation with arsenate stress: 1) As participant in the reduction of arsenate to arsenite, 2) As a substrate for the biosynthesis of phytochelatins, and 3) As chelator of arsenite [19]. At present, the existence of As-metallothioneins and/or phytochelatins in ciliates has not be stated. Our results indicate that, at least in *T. thermophila*, GSH seems to be an important compound involved in cellular defence against As, because GCL gene expression is highly induced (89 folds) after 2h As treatment (Fig. 2).

Zn is the only essential heavy metal that induces significantly (7 folds) the GCL gene expression after a short exposure (2h) (Fig. 2). Likewise, like in filamentous fungi and yeasts [20], starvation induces GSH synthesis in *T. thermophila*.

We might conclude that GSH (the product of the GCL activity) seem to be a universal molecule of general stress, therefore the enzyme GCL might be useful as a molecular biomarker for biomonitoring general stress originated by diverse pollutants.

Acknowledgements This research work was supported by a grant from Ministerio de Educación y Ciencia (Spain), project CGL2005-00548/BOS. Ph.D. scholarship from Universidad Complutense (UCM) is awarded to R.O.

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Identification of culturable psychrophilic yeasts isolated from sediments and melt waters of the Calderone Glacier (Italy)

E. Branda[1], B. Turchetti[1], P. Buzzini[*,1], M. Goretti[1], M. Amici[1], M. Pecci[2], G. Diolaiuti[3], and C. Smiraglia[3]

[1] Dipartimento di Biologia Applicata, University of Perugia, Borgo XX Giugno 74, I-06121, Perugia, Italy
[2] IMONT, Piazza dei Caprettari 70, I-00186 Rome, Italy
[3] Dipartimento di Scienze della Terra "Ardito Desio", University of Milan, via Mangiagalli 34, I-20133, Milan, Italy

[*]Corresponding author: pbuzzini@unipg.it, phone ++390755856455, fax ++390755856470

The present study reports the results of the characterization of psychrophilic yeasts isolated from melt waters and sediments of the Calderone Glacier (Italy). Culturable yeasts, filamentous fungi and bacteria occurring in deep-piping and supraglacial sediments, and in melt waters were monitored. A first set of 30 yeast isolates were identified by using molecular (MSP-PCR fingerprinting and 26S rRNA gene sequencing of the D1/D2 region) methods, as belonging to seven species: *Aureobasidium pullulans*, *Cryptococcus adeliensis*, *Cryptococcus gastricus* (over 50 % of the total strains), *Cryptococcus victoriae*, *Cryptococcus watticus*, *Rhodotorula psychrophenolica* and *Guehomyces pullulans*. One strain, presumably belonging to a new species, yet to be described, was also isolated. The results obtained in the present study suggest the possibility that glacial environments of this European southern glacier could harbor viable psychrophilic yeast populations.

Keywords psychrophilic yeasts; cold environments; glaciers

1. Introduction

Although microorganisms occurring in glacial environments have long been studied exclusively for their ability to survive under such extreme conditions [1-5], more recent studies evidenced that such habitats (e.g. Arctic and Antarctic regions, high mountain glaciers, etc.), can be colonized by obligate and facultative psychrophilic microorganisms [6-9].

Current literature reports the existence of microbial communities in glacier habitats. Viable bacterial populations have been observed beneath glaciers in the northern [7, 10] and southern hemisphere [11]. Besides, de García et al. [12] described the occurrence of viable yeasts in melt waters originating from glaciers of northwest Patagonia, whereas Butinar et al. [13] isolated culturable yeasts from basal ice of an Arctic glaciers. Similarly, Margesin et al. [14] described the occurrence of bacteria and yeasts in Alpine glacier cryoconites, while Buzzini et al. [15] reported the occurrence of culturable yeast cells in melt waters running off from glaciers of Italian Alps. However, despite the above reports, studies on microbial eukaryotic communities occurring in glaciers of southern Europe are so far lacking. The aim of the present investigation has been the isolation and the preliminary characterization of psychrophilic yeasts from melt waters and sediments of the Calderone Glacier (Italy).

2. Materials and methods

After the LGM (Last Glacial Maximum) the Calderone Glacier (Gran Sasso group) represents the last trace of the glaciarized area in the Apennine mountains (Italy). Even if splitted into two ice aprons (usually thin ice mass plastered along a mountain slope or ridge) [16] since the summer 2000, it currently raises a great interest for glaciologists because of its southern geographical location (42°28'15" N) and its environmental features. Its total surface is about 35,000 m^2. Although classified as glacier up to now, because of the lack of any evident dynamic morphology (i.e. crevasses) and any subdivision of the body into two aprons, the Calderone Glacier could be considered an actual *glacieret* (a small ice mass of indefinite shape, on protected slope which originated from snow drifting and/or avalanching; usually no marked ice flow pattern is visible) [16]. The lower and wider ice apron is depressed and completely covered by debris, whereas the upper one is characterized by the presence of seasonal snow.

The samplings have been carried out in the summer of 2006. Due to the high amount of debris covering the glacier surface, and in particular the lower sector and the terminus, the glacier bed beneath the snout area was inaccessible. Accordingly, melt waters was sampled into small glacier holes from which it was possible to reach

the subglacial zone. In addition, also deep-piping sediments (characterized by a grain size between silt and clay) were collected from the glacier surface. All these samples (i.e. water and sediments) originated from subglacial zone and were thus considered representative of the environmental conditions occurring beneath the glacier. Supraglacial sediments (i.e. sediments occurring between rough superficial debris and the top ice surface of the glacier, generally characterized by a grain size from small pebbles and sand) were also sampled. A few Kg of sediments were collected. Since both types of sediments were found to be unfrozen *in situ* when collected, they were stored at 4°C until analysis. Besides, a few liters of melt water was aseptically collected. For all sampling procedures, clean hand tools were surface sterilized by using 70% ethanol immediately before use, and between each sampling. Sterile containers were used for sample storage. Samples were stored at 4°C until analysis.

Sediments and melt waters were analyzed for dry weight (DW) and pH [17, 18]. Viable counts of yeasts and filamentous fungi were carried out by using Rose Bengal agar (RB) + tetracycline (Difco, USA). Plate Count agar (Difco) was used for bacterial counts. Melt waters were filtered through 0.22 μm filters (Millipore, USA), whereas sediments were serially diluted with sterile 0.1% sodium pyrophosphate. Both were plated onto Petri dishes containing the above media and then incubated at two different temperatures (4 and 20°C for 12 or 3 weeks, respectively). All chemical and microbiological analyses were carried out in triplicate and statistical evaluation of average values was carried out by ANOVA.

Colonies grown on the plates were periodically checked, and transferred to RB without tetracycline for purification. They were selected for isolation on the basis of their morphology, paying attention to isolating all types occurring at the different incubation temperatures. All yeast strains isolated are deposited in the Industrial Yeasts Collection DBVPG of the University of Perugia (Italy) (www.agr.unipg.it/dbvpg).

Total genomic DNA extraction was done according to Sampaio et al. [19]. The synthetic oligonucleotides $(GTG)_5$ and M13 (MWG Biotech, Germany) were used as single primers for MSP-PCR fingerprinting. All PCR reactions were performed, according to Meyer et al. [20]. DNA amplification was performed in a T personal Combi Thermal Cycler (Biometra Gmbh, Germany) by using the following PCR program: i) initial denaturing step at 95°C for 5 min; ii) 40 cycles of 40 s at 95°C, 1 min at 50°C and 1 min at 72°C; iii) final extension step at 72°C for 5 min. A negative control was also included in all PCR reactions. Amplification products were separated by electrophoresis in 1.4% w/v agarose gels containing ethidium Bromide in 1 X TAE (Tris-Acetate-EDTA) buffer at 120 V for 1.5 h. Gels were stained with ethidium bromide. Electrophoretic bands were visualized under UV light. DNA banding patterns were analyzed by using the Image software package. In agreement with current literature [19, 21], strains exhibiting identical DNA-banding patterns were grouped, and considered to belong to the same species.

Representative strains from each of the groups were subjected to sequencing of the D1/D2 domain of 26S rRNA gene. DNA was first amplified using the primers ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G) and LR6 (5'-CGC CAG TTC TGC TTA CC) (MWG Biotech). A 600-650 base pairs region was sequenced by the forward primer NL1 (5'-5'-GCA TAT CAA TAA GCG GAG GAA AAG) and the reverse primer NL4 (5'-GGT CCG TGTTTC AAG ACG G) (MWG Biotech). Sequences were obtained by an Applied Biosystems DNA Sequencer, mod. ABI PRISM 377 (Applied Biosystems, USA) using standard protocols. Alignments were made by using Vector NTI Suite 8 Contig Express (Informax, Invitrogen). Strains were identified by comparing the sequences obtained with the GenBank database (BLASTN freeware from www.ncbi.nlm.nih.gov/BLAST).

3. Results and discussion

The pH of melt waters and sediments were similar to one another (Table 1). Concerning dry weight (DW), no significant ($p < 0.01$) differences have been observed between supraglacial and deep-piping sediments (Table 1).

Table 1 pH and dry weight (DW %) of sediments and melt waters collected in the Calderone Glacier (Italy). All determinations were carried out in triplicate.

	supraglacial sediments	deep-piping sediments	melt waters
pH	7.3	8.4	7.7
DW %	68.8%	83.5%	0.1%

Culturable yeasts in supraglacial sediments ranged from 10^3 to 10^4 CFU g^{-1} DW. On the contrary, the number of yeast cells in deep-piping sediments was 10^2 CFU g^{-1} DW. Melt waters contained a number of culturable yeast cells from 70 to 10^2 L^{-1} (Table 2). Culturable filamentous fungi in supraglacial and in deep-piping sediments were from 10^3 to 10^5 CFU g^{-1} DW. In contrast, melt waters contained a few hundreds of CFU mL^{-1} (Table 2). The viable counts of culturable bacteria gave results of several order of magnitude higher than that

found for yeasts and filamentous fungi. In supraglacial and in deep-piping sediments, a number of viable bacterial cell from 10^5 to 10^6 g^{-1} DW were observed, whereas melt waters contained 10^4 CFU L^{-1} (Tables 2).

Table 2 Enumeration of yeasts, filamentous fungi and bacteria in sediments and melt waters collected in the Calderone Glacier (Italy). All determinations were carried out in triplicate. DW = dry weight.

		supraglacial sediments (CFU g^{-1} DW)	deep-piping sediments (CFU g^{-1} DW)	melt waters (CFU L^{-1})
viable count at 4°C	yeasts	1.4×10^4	5.0×10^2	2.2×10^2
	filamentous fungi	1.3×10^5	4.5×10^3	516
	bacteria	1.8×10^6	2.2×10^5	1×10^4
		(CFU g^{-1} DW)	(CFU g^{-1} DW)	(CFU L^{-1})
viable count at 20°C	yeasts	6.1×10^3	2.5×10^2	70
	filamentous fungi	1.5×10^5	4.0×10^3	300
	bacteria	1.7×10^5	1.7×10^5	1×10^4

A first set of 30 yeast isolates were characterized by MSP-PCR fingerprinting as belonging to 15 distinct profiles, 5 of which were shared by at least two strains which formed groups of identical DNA-banding patterns (Table 3). All sequences obtained in this study were deposited in the GeneBank database (www.ncbi.nlm.nih.gov/BLAST) and their accession numbers are shown in Table 3.

The 26S rRNA gene D1/D2 region sequence analysis of representative strains from 14 out of 15 MSP-PCR profiles allowed them to be assigned to the following species: *Aureobasidium pullulans*, *Cryptococcus adeliensis*, *Cryptococcus gastricus* (over 50 % of the total strains), *Cryptococcus victoriae*, *Cryptococcus watticus, Guehomyces pullulans* and *Rhodotorula psychrophenolica*.

A few species (*C. gastricus, C. victoriae* and *G. pullulans*) were characterized by heterogeneous MSP-PCR profiles, whereas the 26S rRNA gene D1/D2 sequence of one yeast strain, included within the MSP-PCR patterns labeled with the number 12, was characterized by 99% similarity with sequences (deposited with the number AY040646) belonging to so far unidentified strains labeled as "Antarctic yeasts" (Table 3) and its formal taxonomic designation is in progress.

Yeast biodiversity observed in sediments and melt waters of the Calderone Glacier has been compared with that previously observed in other glacial environments. *A. pullulans* and *R. psychrophenolica* have been previously isolated from high Arctic and Alpine glaciers [13, 22-24].

Table 3 Clustering of yeast isolated from the Calderone Glacier (Italy) to MSP-PCR fingerprinting patterns and Gene bank accession numbers for D1/D2 domain sequences of the 26S rRNA gene. [1] = different numbers (1-15) indicate different MSP-PCR fingerprinting patterns; [2] = GenBank accession numbers of sequenced strains; [3] = percentages of sequence similarity after Blast analysis.

DBVPG accession number	Groups[1]	Species	GenBank D1/D2 26S rRNA gene[2]	GenBank D1/D2 26S rRNA gene[3]
4838, 4839	1	*Cryptococcus gastricus*	EU287887	100% to AF137600
4816, 4818 4821, 4823 4825, 4829 4833, 4834 4840, 4842	2	*Cryptococcus gastricus*	EU287889	100% to AF137600
4820, 4832 4843	3	*Cryptococcus gastricus*	EU287878	100% to AF137600
4824, 4827 4831	4	*Cryptococcus gastricus*	EU287883	100% to AF137600
4826	5	*Cryptococcus victoriae*	EU287880	99% to AF363647
4830	6	*Cryptococcus victoriae*	EU287882	100% to AF363647
4835	7	*Cryptococcus victoriae*	EU287884	99% to AF363647
4822	8	*Guehomyces pullulans*	EU287879	100% to AF105394

4836	9	*Guehomyces pullulans*	EU287885	100% to AF105394
4845	10	*Cystofilobasidium capitatum*	EU287890	100% to AF075465
4817, 4844	11	*Rhodotorula psychrophenolica*	EU287876	99% to EF151255
4841	12	unidentified Antarctic yeast	EU287888	99% to AY040646
4837	13	*Cryptococcus watticus*	EU287886	99% to AY138478
4819	14	*Cryptococcus adeliensis*	EU287877	99% to AF137603
4828	15	*Aureobasidium pullulans*	EU287881	99% to EF690465

A few species (*C. victoriae*, *C. watticus* and *C. adeliensis*) were previously found in Antarctic habitats [25-27]. Similarly, the unidentified yeast species found in this study exhibited 100% similarity with 26S rRNA gene D1/D2 sequence of a yeast strain originating from Antarctica (Table 3). Besides, *C. adeliensis* and *Cystofilobasidium capitatum* have been previously isolated from melt waters of Patagonian glaciers and from frozen environmental samples in Iceland, respectively [12, 28].

Finally, the species *C. gastricus* and *G. pullulans* have been previously found to be soil-inhabiting yeasts in New Zealand [29] Korea [30] and Japan [31]. This is the first study reporting their isolation from glacial environments.

Acknowledgements The support by MIUR (PRIN Project 2006-2007) is gratefully acknowledged.

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In situ assessment of drinking water biostability using nascent reference biofilm ATR-FTIR fingerprint

F. Humbert[*], **F. Quilès,** and **A. Delille**

Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, Nancy University, CNRS, 405, rue de Vandoeuvre, F-54600 Villers-lès-Nancy, France

[*]Corresponding author: e-mail: francois.humbert@lcpme.cnrs-nancy.fr, Phone: +33 383685238

Drinking water biostability is commonly assessed by biomass-based or biodegradability methods. While these methods are often effective, they suffer from several limitations in particular long analysis time or/and batch processing. Moreover, culture-based methods do not reflect the actual dynamic of the attached bacterial films on pipe surfaces, which play a major role in post-disinfection biological regrowth. Consequently, in addition to these methods, managing finished water quality requires new early warning devices which enable to detect quickly, or even to forecast, degradation of drinking water microbial quality during its transport throughout distribution systems. For this purpose we have recently suggested to monitor, *in situ* and in real time, drinking water biostability using a biosensor based on Attenuated Total Reflectance - Fourier Transform InfraRed fingerprint of a nascent reference biofilm consisting of a monolayer of bacteria. The principles and the potential of this approach are illustrated through several examples. Current limitations of the method and the future challenges such as the implementation of such a biosensor in real drinking water systems are discussed.

Keywords drinking water; biostability; ATR-FTIR spectroscopy; nascent biofilm; *Pseudomonas fluorescens*

1. Introduction

The microbial quality of drinking water can be significantly deteriorated during storage and transport in distribution systems for many reasons, but especially due to mechanical failures, which result in introduction of bacteria into the distribution network, and the formation of biofilms associated with bacterial regrowth [1]. Indeed, disinfection of drinking water does not produce a sterile product. Bacteria can survive the treatment process and utilize nutrients in water to growth or regrowth. In a distribution system, most of bacteria attach to pipe or reservoir inner surfaces where they form biofilms. Biofilms are clusters of microorganisms and inorganic or organic materials held together by a matrix of organic polymers and attached to a substratum such as pipes. These biofilms can subsequently seed the water phase with bacteria, but also shelter various pathogens, promote development of some of them, and offer protection from biocides. The ability to limit bacterial regrowth in drinking water is referred to as biological stability or biostability. It is dependent on a large number of parameters, including temperature, pH, disinfectant residual, hydrodynamic conditions, residence time, pipe materials, and above all the availability and composition of biodegradable organic matter [1]. Drinking water biostability is commonly assessed by biomass-based or biodegradability methods [2-3]. The standard methods rely upon the determination of biodegradable dissolved organic carbon (BDOC) and assimilable organic carbon (AOC) [2]. While these methods are often effective, they suffer from several limitations. In particular, they entail long analysis time (usually more than 24 h), or/and batch processing, and do not reflect the actual dynamic of the attached bacterial films on pipe surfaces. Moreover, depending on treatment, the standard organic carbon biodegradability measurements (assimilable, biodegradable dissolved and dissolved organic carbon) can give contradictory results as to the biostability of given water. Indeed, many factors other than organic carbon can promote or limit microbial growth [2, 4]. To more reflect actual dynamic of biofilms in distribution system, the standard methods (AOC and BDOC) may be used in conjunction with biofilm formation potential methods [5]. But these latter methods require the removal of biofilms for various *ex situ* analysis. Consequently, in addition to these methods, managing finished water quality requires early warning devices to quickly detect *in situ*, or even to predict, deterioration of drinking water quality. Since the development of biofilms is a key factor in drinking water biostability and depends on various environmental parameters, a possible strategy for assessing drinking water biostability could consist in monitoring continuously, *in situ* and in real time, the status of biofilm development on the inner surfaces of the distribution system. Among the possible techniques for monitoring in situ biofilms, there is the attenuated total reflection – Fourier Transform Infrared (ATR-FTIR) spectroscopy.

2. *In situ* biofilm monitoring by ATR-FTIR spectroscopy

The IR spectroscopy is a vibrational spectroscopy which provides quantitative information about the chemical composition of a sample. Infrared spectra of bacteria show various bands which can be assigned to the main groups of biomolecules, namely proteins, nucleic acids, polysaccharides, and lipids (Fig. 1). In ATR mode, the sample is placed in contact with a crystal of high refractive index, superior to the sample refractive index (Fig. 1). The infrared beam is focused onto the edge of the ATR crystal, multiply reflected on the inner surface of the crystal, and then directed to a suitable detector. At each reflection at the sample – ATR crystal interface, an infrared evanescent wave is created into the sample where it can be absorbed, allowing obtaining the infrared fingerprint of the sample. The electric field amplitude of this evanescent wave decaying exponentially with distance from the crystal surface, the analysis depth is very thin, typically of the order of 1-2 µm [6].

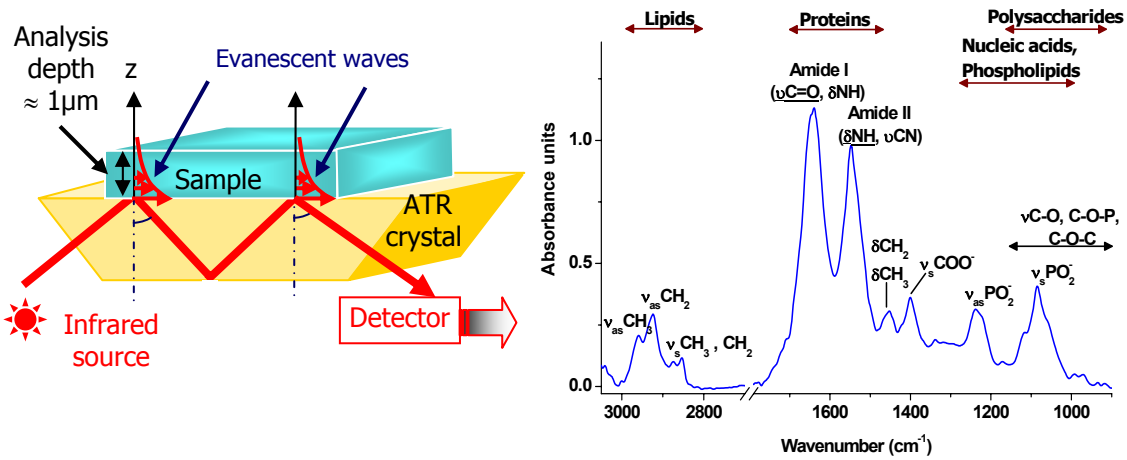

Fig. 1 Principle of the ATR-FTIR technique and ATR-FTIR fingerprint of *Pseudomonas fluorescens* planktonic bacteria harvested at end-exponential phase. The main assignments are indicated. ν_s , ν_{as} symmetric and asymmetric stretch, δ deformation vibration. For more details see [7].

This last feature is both a drawback, only the base layer of biofilms being analyzed (at least in this configuration), and a strength of the technique which thus allows to monitor *in situ* the initial stages of biofilm formation as perfectly exemplified in figure 2. This figure shows the spectral changes occurring in the course of the ATR crystal colonization by *Pseudomonas fluorescens* bacteria and subsequently the gradual biofilm development when a nutritive medium is alone pumped through the ATR flow cell. For more details about the experimental conditions see reference [7]. The time evolution of the band mainly assigned mainly to proteins, named amide II, allows monitoring biomass accumulation on the ATR crystal, whereas the detailed analysis of the whole spectral profile provides information about chemical changes accompanying bacterial adhesion and biofilm development processes. Thus, the intensity of all bands increased over the 3h inoculation period reflecting an accumulation of biomass on the ATR crystal surface and an increase of the crystal surface coverage by bacteria. After 3 h of inoculation, the intensity of all bands continued to increase but the polysaccharide band increased at a higher rate than the amide II band mainly associated with proteins. This increase reflected biofilm development, bacterial cells dividing on, colonizing the IRE surface and producing extracellular polymeric substances. At about 6h, a biofilm was really formed and consisted of a monolayer of bacteria as confirmed by the epifluorescence image, hence the expression of "nascent biofilm". Then, the IR band intensity still continued to increase during about 8 hours but significant changes occurred in the spectral profile reflecting structural and compositional changes in the biofilm. For example, from 15h, a glycogen production was observed. This experiment type allowed determining the influence of nutrient stress on the initial stages of *Pseudomonas fluorescens* biofilm formation, in particular in drinking water [7].

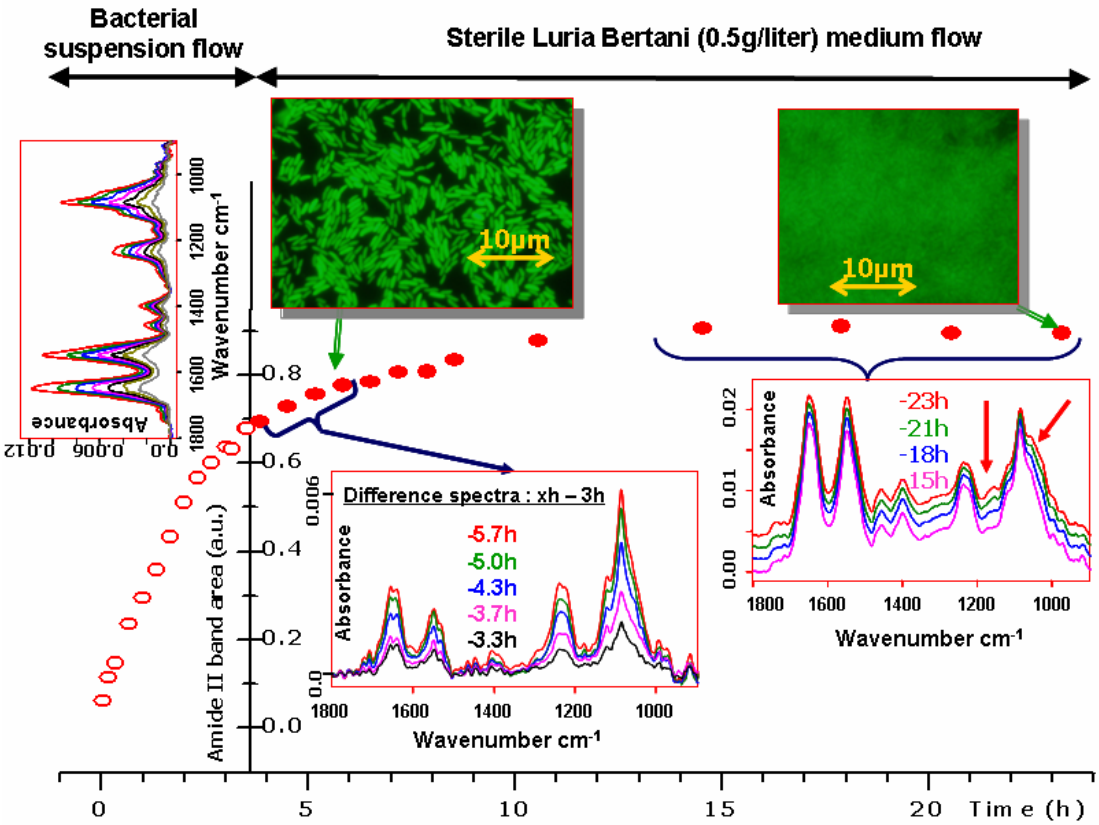

Fig. 2 Time evolution of ATR-FTIR spectra and the amide II band intensity during the initial stages of *P. fluorescens* biofilm formation on a germanium ATR crystal. A suspension containing end-exponential-phase bacteria (~10^8 CFU/ml) in sterile LB medium (0.5g/liter) was introduced into the flow cell at time zero and flowed through the cell for 3 h. Subsequently, sterile LB medium was pumped through the flow cell for 24 h. The epifluorescence microscope images show the *P. fluorescens* biofilm at 3h and 25h respectively. a.u.: arbitrary units. For more details, see reference [7].

However interesting these studies are, they carried out only under laboratory and not field conditions. The implementation of such a technique for assessing water biostability at any point of a distribution system comes up against many difficulties, besides cost and technical aspects. Firstly, considering the thinness of the analysis depth, the necessary heterogeneous nature of deposit, from drinking water, on the ATR crystal surface makes it difficult to detect bacteria with a good signal/noise ratio. Secondly, in the case of biofilm formation, its response time to environmental condition changes may largely vary according to bacterial strains forming biofilm. In addition, this response may be not representative of the global microbial quality of water in the distribution system.

3. Response of the 6-h *P. fluoresens* nascent biofilm to changes in dissolved organic carbon level

To circumvent these difficulties a possible alternative could consist of developing a biofilm-type biosensor. The biological recognition element should be a monolayer reference biofilm in contact with an ATR crystal which should be the transduction element. The water biostability assessing should rely on the analysis of the changes in the ATR-FTIR fingerprint of the nascent reference biofilm. A first exploratory study of such a system has been recently published [7]. In this study, a 6h nascent *Pseudomonas fluorescens* biofilm has been used as the biological recognition element. As shown by the epifluorescence image and the IR spectral region assigned to polysaccharides (Fig. 2), under experimental conditions of the study, a 6h *P. fluorescens* biofilm consist of only a monolayer of bacteria embedded in a matrix of extracellular polymeric substances (EPS). Dissolved organic carbon (DOC) level being a key factor of the bacterial regrowth in drinking water, the biosensor response to variations in DOC level in tap water has been monitored over several days.

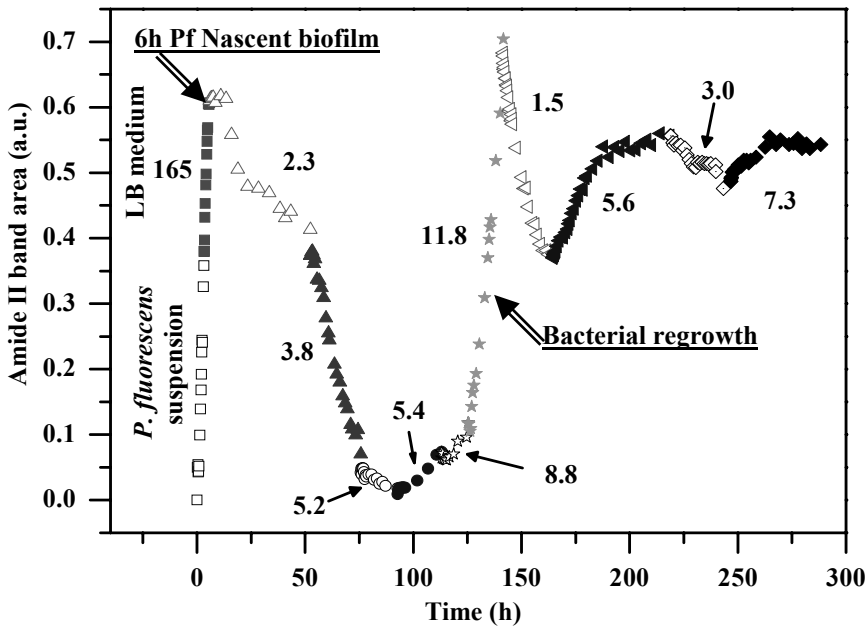

Fig. 3 Effect of DOC level changes on nascent *P. fluorescens* biofilm development monitored by changes in the amide II band area. The nascent biofilm attached to the Ge ATR crystal and resulting from 3-h inoculation period (open squares), and 3h sterile LB flow period (closed squares), was subjected to a series of dechlorinated sterile drinking water samples containing various DOC levels under flow conditions. DOC level (mg/liter) is indicated for each water sample. (Delille et al., 2007).

As highlighted in figure 3, any variation in DOC level modified the kinetic of nascent biofilm development within 1-2 hours. Biofilm detachment and regrowth kinetics determined from changes in the area of infrared bands associated with proteins and polysaccharides were directly dependent upon DOC level. In addition, they were consistent with bacterial adhesion or growth kinetic models and the EPS overproduction or starvation-dependent detachment mechanisms. These findings highlight perfectly the potential of the suggested approach regarding finished water management, allowing for example, in the present study, to estimate health risk entailed by biofilm detachment resulting from DOC level variation and consequently to quickly apply appropriate counter measures. Biosensor early warning capacity is mainly due to the reference biofilm quality of being nascent. A further advantage against the traditional methods is that any factor promoting a significant regrowth or detachment of the nascent reference biofilm, can be detected even if this factor is below detection limits of conventional analytical techniques. However, this study, carried out under laboratory conditions, is only the first step toward the development a biosensor for assessment of biostability in real drinking water distribution system. Much research is needed to confirm and extend these findings. In particular it is necessary to optimize biosensor sensitivity and evaluate the effect of other parameters such pH, temperature, disinfectant concentration, hydrodynamic conditions, and the nature of organic matter present in drinking water on the response of nascent reference biofilm. Other microbial strains as well as strain mixtures should be also consider as biological recognition element. In addition, chemometric analysis could aid in interpretation of spectral changes and consequently in monitoring automation, and decision making. Finally, the use of optical fibers operating in mid-infrared region, such as chalcogenide glass fibers, should allow considering implementation of such biosensors at any point of a distribution system.

4. Conclusion

To date, no device allows early detecting *in situ* or predicting deterioration of water microbiological quality in a drinking water distribution system. Such an early warning device should yet be a valuable decision making tool for water utilities. To fill this lack, considering the key function of biofilms in water biostability, the best strategy is to develop a system capable of monitoring continuously, *in situ* and in real time, the status of biofilm development within distribution systems. This devise must be capable to detect changes not only in biomass amount but also in biofilm chemical composition and physiological state. In addition, to assess biofilm formation potential it must be sufficiently sensitive to monitor the initial stages of biofilm formation and consequently, to detect a bacteria monolayer. These criteria could be met by a biofilm-type biosensor consisting of a monolayer reference biofilm in contact with an ATR crystal which should be the transduction element. The

water biostability assessing should rely on the analysis of the changes in the ATR-FTIR fingerprint of the monolayer reference biofilm. This study supports this approach but more research and development is still needed before considering implementation of such a biosensor in real drinking water systems.

Acknowledgements This work has been undertaken as part of a research project which is supported by the European Commission within the Fifth Framework Programme, "Energy, Environment and sustainable development programme", no EVK1-2002-00108. Disclaimer stating that the authors are solely responsible for the work, it does not represent the opinion of the Community and the Community is not responsible for any use that might be made of data appearing therein.

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Influence of light: dark cycle in the cellular composition of *Nannochloropsis gaditana*

M.H.F. Henriques[*,1] and **J.M.S. Rocha**[2]

[1] Escola Superior Agrária de Coimbra, Polytechnic Institute of Coimbra, Bencanta, 3040-167 Coimbra, Portugal
[2] CIEPQPF, Chemical Engineering Department, University of Coimbra, Rua Sílvio Lima - Pólo II, 3030-790 Coimbra, Portugal

[*]Corresponding author: e-mail: mhenriques@esac.pt, Phone: +351 239 802 940

Nannochloropsis gaditana is recognized as a source of valuable pigments and polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA, 20:5ω3). The duration of the light period is an important environmental factor that affects the production yield in photosynthetic microorganisms. This factor was found to determine the growth and biochemical composition of this microalga strain. Cell division occurs during the first hours of the light period. Whereas carbohydrates are accumulated in the light period and consumed at night, the cellular concentration of chlorophylls and proteins does not vary significantly. The storage lipids are consumed in the lag phase (after dilution of the culture) and they increase until the final growth period. The structural lipids reach a maximum at the end of the daily exponential phase, when the cells also reach a maximum density, in a semi-continuous culture with a daily replacement of fresh medium.

Keywords microalgae; *Nannochloropsis gaditana*; light:dark cycles; biochemical composition

1. Introduction

Microalgae are adapted to the environmental conditions in their natural habitat, especially to the availability and intensity of light during the illuminated period. Apart from diatoms, many species carry out cytokinesis during a restricted period of time. This period generally corresponds to the dark phase, since the cell's periodic responses tend to synchronize cell division [1]. The control of cell division by the light:dark cycle influences the cell's biochemical composition, namely the pigment, protein, carbohydrate and fatty acid contents [2]. During the daily cycle not only the main pigment concentrations vary, but also the secondary pigment contents and the phototrophic activity of the cells [3]. The daily biochemical evaluation of the culture in a pseudo-steady state showed a progressive increment in cellular components, such as pigments and fatty acids during the entire light phase, but there were only local increases for proteins and carbohydrates [1]. Due to the commercial interest in *Nannochloropsis gaditana* for aquaculture, the aim of this work is the evaluation of the influence of a daily harvest of cells in the enrichment of the microalgal biomass.

2. Material and methods

2.1 Growth conditions

Cultures of *Nannochloropsis gaditana* were obtained from Necton (Portugal). The microalga was grown in synthetic seawater medium in Erlenmeyer flasks with cotton covers before inoculation into tubular bubble reactors. The composition of the medium was previously described [4].

Tubular bubble reactors (glass tubes with 70 cm length and 5 cm diameter with a culture volume of 0.7 L) were sparged with air at 120 mL/min flow rate, kept at 18±1 ºC and illuminated by an average of 15 μmol photons m^{-2} s^{-1}, provided by natural sunlight. Once the cultures reached the early stationary phase, the semi-continuous regime was started with a daily renewal at 11 a.m. of 7% of the culture volume using sterilized medium with the same initial composition.

2.2 Analytical methods

The cell concentration was evaluated by measuring the optical density of the culture at 540 nm in a Beckman DU Series 600 spectrophotometer. The absorption at 540 nm was correlated to the cell dry weight and to the cell number per millilitre of culture. The content of total carbohydrates was measured by the colorimetric phenol-sulphuric acid method [5]; chlorophyll *a* (chla) was evaluated by the Mackinney method [6] and total proteins were determined by the Lowry method modified by Peterson [7]. The fatty acid methyl esters (FAME) were

prepared by transesterification of lyophilized cells, according to the Sato and Murata method [8], using nonadecanoic acid (C19:0) as an internal standard. FAME samples were analyzed using a TREMETRICS 9001 chromatograph with a FID detector using a DB225 J&W SCIENTIFIC capillary column (30 m length, 0.25 μm internal diameter and 0.15 μm film thickness). The oven time-temperature program was the following: 70 ºC (1 min), 20 ºC/min until 180 ºC, 5 ºC/min until 220 ºC (5 min) and 4 ºC/min until 240 ºC (2 min), giving a total running time of 27 min. Fatty acids were identified by comparison to the retention times of known standards obtained from Sigma. All analyses were made in triplicate.

3. Results and discussion

3.1 Cell Growth

The cultures were grown for 20 days in batch regimen until the stationary phase was reached. The cell concentration achieved was approximately 45×10^6 cell/mL. After 15 days following a semi-continuous regimen a pseudo-steady state was achieved and maintained for seven days, when the physiological measurements were performed.

The cell concentration just before each daily dilution was 16×10^6 cell/mL (Fig. 1). After dilution, the cell concentration was evaluated at half an hour for the first time and every two hours during the light period. After the removal of part of the biomass at 11 a.m. the cell concentration in the culture was quite constant until the end of the dark period (7 a.m. of the following day). At the beginning of the following light period the cell density reached the same value as before the removal of biomass. These results contradict the ones obtained by some authors that reported cell division only occurs during the beginning of the dark period [1, 9]. Figure 1 shows that cell division is synchronized and occurs during a very restricted period of illumination (the first 4 hours), although a longer cell division phase (8 h) has been reported [9] and a cell growth phase of only one hour long has been reported in [1]. Usually, cell division only occurs once during the light:dark cycle, and this results in the synchronization of cell division in the phytoplankton population. The maximum light intensity that reached the cultures during our study was 27 μmol photons m^{-2} s^{-1}, which is a very modest value and may have been a growth-limiting factor.

3.2 Biochemical composition

The concentration of chla in the culture volume was almost constant during the light period. However, the chlorophyll a content per cell showed a marked variation during the same period (Fig. 2a). This phenomenon can be explained as a cellular response to the light intensity and photosynthetic yield. After the dark period (when the light intensity was minimal) the chla content in cells increased (Fig 2a) in order to improve the photosynthetic efficiency. When the light intensity begins to increase the chla content decreases again. This way, the chlorophyll concentration per cell may adjust without any restriction in the photosynthetic process. Cells use light energy for the production of other fundamental compounds during the growth process, taking profit of pigments. It has also been reported that chlorophyll a and carotenoids increase throughout the light period, although the rise in pigment production rate only occurs after eight hours of illumination [1]. In this study, this was not observed (Fig. 2a), perhaps due to the low cell concentrations. The dilution effects have a stronger influence when cell densities are large, but in this case the shadow effects were not limiting.

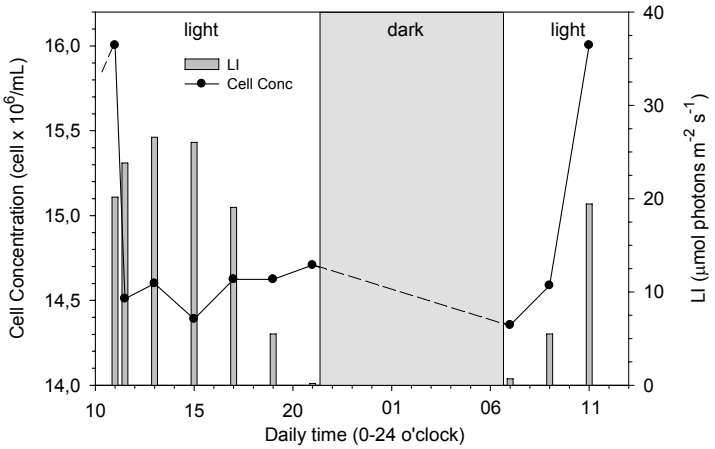

Fig. 1 *Cellular concentration and light intensity (LI) during a 14:10 h light:dark cycle with natural illumination.*

The maximum carbohydrate content (4.2 pg cell^{-1}) occurred at the end of the illuminated period, followed by a decrease to a minimum of 2.3 pg cell^{-1}. Because these compounds are the most accessible energy source for cellular metabolism, this decline during both the dark and the growth phase is inevitable (Fig. 2b).

During the light period, the protein content did not vary significantly (Fig. 2c); only at the end of the growing phase a maximum of 4 pg cell^{-1} was achieved (in the literature [1] a maximum of 3 pg cell^{-1} is referred). In this study the protein content was shown to double during the light:dark cycle. Authors that have estimated the protein content using the nitrogen composition, applying an elemental analysis, found values slightly higher than the ones obtained using the Lowry method. However, in both cases the evolution during the daily cycle was similar. The divergence between the two methods may be due to the influence of some nitrogen residues present in the samples, which leads to higher values in the elemental analysis, or by the intrinsic limitations of the Lowry method [10] and its interferences in colour development.

At the end of the stationary phase the total fatty acids represent 40% of the dry biomass. The fatty acid accumulation/concentration occurs during the night period as a result of the consumption of carbohydrates used as energy source for cell maintenance. The variations in fatty acid composition during the light:dark cycle can be related to exchange between structural lipids (e.g. galactolipids) and reserve lipids (e.g. triacylglycerols) as shown in Fig. 3. The reserve lipids in *Nannochloropsis gaditana* (C16:0 and C16:1) were concentrated during the night period and consumed in the acclimation phase, c.a 6 hours after the partial removal of medium (Fig. 3). The relative mass concentration of each compound is obviously affected by the instantaneous content of all other components in the cell. The content of structural lipids (C20:5n3 and C20:4n6) increases during the dark period [2] but in the acclimation phase is relatively constant, reaching a maximum at the end of the exponential phase when the number of cells is also the highest one in the daily cycle. The relative proportion between reserve lipids and structural lipids (Fig. 3) decreases after the partial medium removal and it reaches a minimum by the end of the illuminated period.

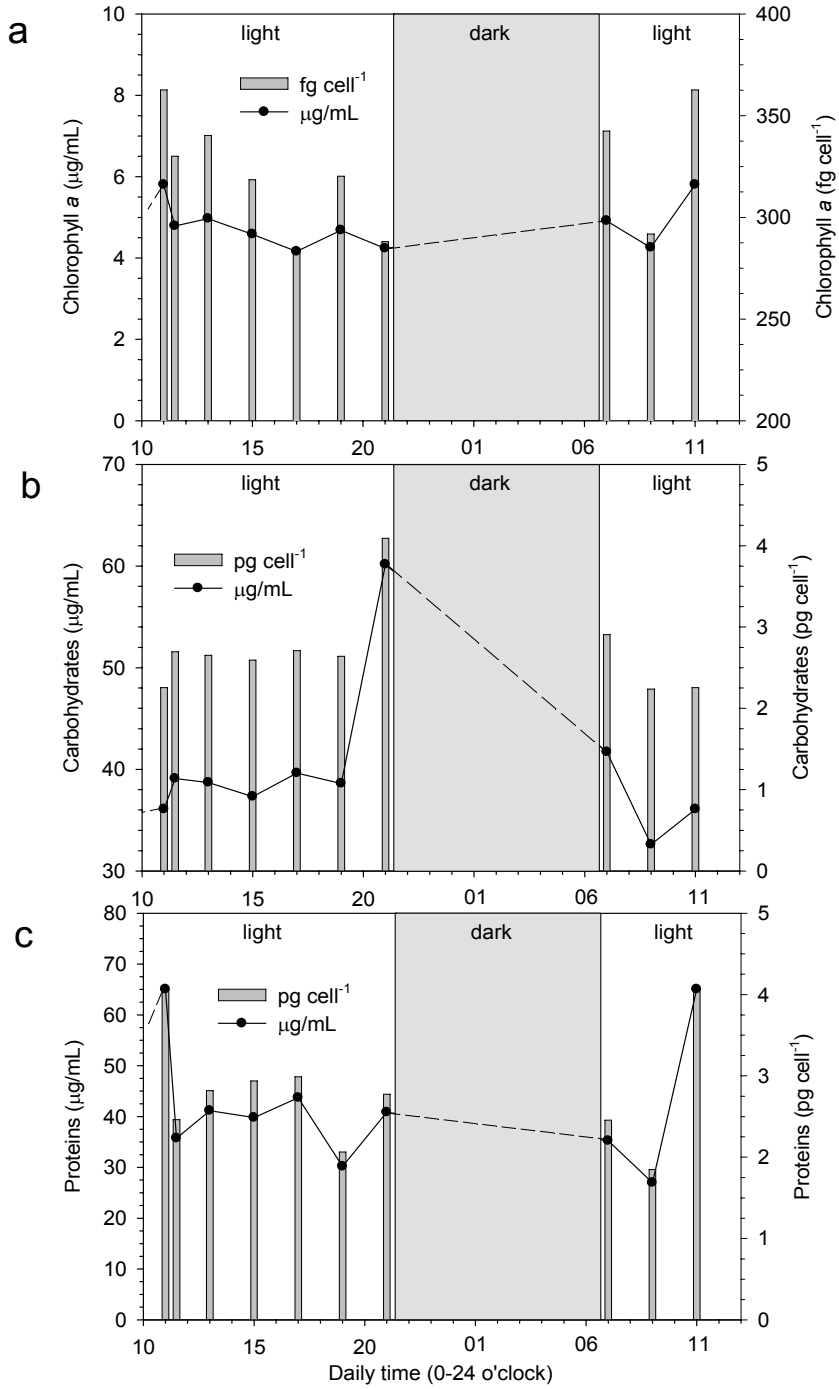

Fig. 2 *Biochemical composition during a 14:10 h light:dark cycle with natural illumination: (a) chlorophyll a content (μg/mL); (b) carbohydrate content (μg/mL);(c) protein content (μg/mL).*

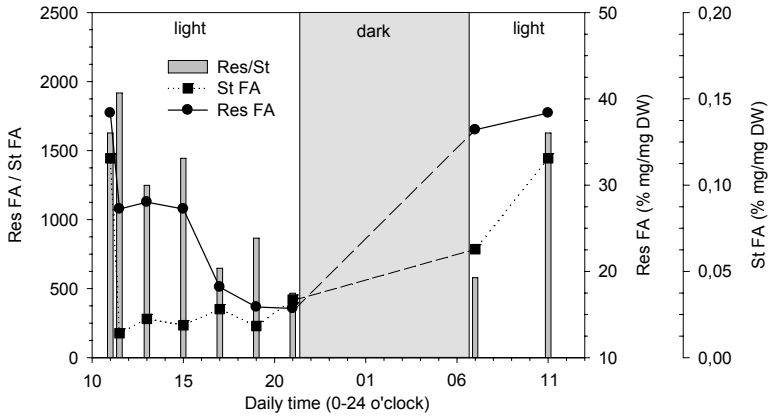

Fig. 3 *Fatty acid composition during a 14:10 h light:dark cycle with natural illumination. St FA – structural lipids; Res FA – reserve lipids.*

4. Conclusions

Under optimized culture conditions, both to the cell growth itself or to the production of any interesting metabolite, the largest productivities are obtained with a semi-continuous operation mode. In this culture strategy a volume of culture can be periodically harvested and replaced by the same amount of fresh medium, and a pseudo steady-state can be reached. However, with photosynthetic organisms in natural light environment also the daily time of the cells harvest influence the concentration of the target compound and, consequently, the production yield of the desired product. *Nannochloropsis gaditana* must be harvested by the end of the sun day (illuminated period) if the nutritional composition of the cells (namely carbohydrates) must be preserved. However, if the content of pigments is the target goal, such as chla, to increase the green colour intensity (e.g. to make microalgal biomass more attractive in aquaculture), harvesting must be done early in the morning, when pigments are more concentrated within the cells. On the other hand, if cell density must be maximized, or the structural lipids (polyunsaturated fatty acids associated to cell membranes) must be favoured over the reserve lipids, the harvesting must be done 3-4 h after the beginning of the illuminated period, to take profit of the daily cell multiplication.

Acknowledgements To the Necton Company for the kind supply of the *Nannochloropsis gaditana* strain used in this study.

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Isolation and Identification of Bacteria in the Anammox Activated Sludge

Qin Yu-jie*, Zhou Shao-qi

College of Environmental Science and Engineering, South China University of Technology, Guangzhou 510006, China

*Corresponding author：E-mail: qinyujie@scut.edu.cn

The anaerobic ammonium oxidation (anammox) microbe in an upflow anaerobic sludge blanket (UASB) bioreactor was successfully enriched under inorganic and dark condition. Seven species were isolated and purified in conventional methods from the activated sludge. Based on the morphological, physiological, biochemical and 16S rRNA analysis, these bacteria belonged to *Pseudomonas sp*, *Clostridium beijerinckii*, *Bacillus cereus*, two kinds of *Bacillus* sp., *Sphingosinicella microcystinivorans* and unidentified bacterium. The cells concentration of E1 strain (*Pseudomonas sp*) (>10^7cell/ml) was the highest of these seven kinds of bacteria. The anammox activities of the seven strains were evaluated. Only E1 strain made ammonium oxidize in anaerobic condition. E1 was a denitrifying bacterium and had some anammox activity.

Keywords enrichment;isolation; identification; *Pseudomonas sp*; Anammox; denitrogenation

1. Introduction

Anammox is a fascinating microbial pathway. In the anammox process, chemolithotrophy autotrophic bacteria convert 1 mol of nitrite and 1 mol of ammonium directly to dinitrogen gas with hydrazine as an intermediate [1]. Compared to conventional nitrification-denitrification-dependent nitrogen removal systems, it is a promising process of biological nitrogen removal in wastewater treatment due to its unique feature such as with no additional organic compound, without consuming oxygen, and without producing secondary pollution in the process of reaction[2]. The uncovered process can save up to 90% of operation cost as compared to typical nitrogen treatment processes[3]. This process is now applied in low-cost N-removal from wastewater without the addition of organic carbon in a full-scale reactor in Rotterdam, the Netherlands [4].

Till now only anammox species belonging to *Canditatus* “Scalindua sorokinii” was detected in natural saline ecosystems [5]. The other five species which are named temporarily *Candidatus* “Kuenenia stuttgartiensis”, “Brocadia anammocidans”, “Scalindua wagneri”, “Scalindua brodae”and “anammoxoglobus propionicus” respectively are known to exist in freshwater ecosystems [6-9]. Anaerobic ammonium oxidation (anammox) bacteria are still not purified, and their characteristics are still not known well. Most scientists think the bacteria carrying out the anammox pathway belong to the planctomyces [6]. Hu found that other genus bacteria except planctomyces might have the anammox activity [10]. A major aim of the research was to find new anammox active bacteria. To achieve this goal we enriched the anammox bacteria in an upflow anaerobic sludge blanket (UASB) reactor, and isolated and purified the bacteria from the activated sludge.

2. Material and Methods

2.1 Anammox bacteria enrichment

An UASB reactor was used for anammox enrichment. The reaction was kept at 32-34ºC. The influent of mineral medium used for anammox bacteria enrichment was synthetic wastewater. The influent composition of the mineral medium was as follows (g/l): $KH_2PO_4$0.027, $MgSO_4{\cdot}7H_2O$ 0.3, $CaCl_2$ 0.136, $NaHCO_3$ 0.5, NH_4Cl 0.11 -0.38 and $NaNO_2$ 0.15-0.49; Its microelement was as follows (mg/l): EDTA 5, $FeSO_4$ 5, $ZnSO_4{\cdot}7H_2O$ 0.43, $CuSO_4{\cdot}5H_2O$ 0.25, $MnCl_2{\cdot}4H_2O$ 0.99, $NiCl_2{\cdot}6H_2O$ 0.19, $CoCl_2{\cdot}6H_2O$ 0.24 and H_3BO_4 0.014. The pH value of influent was adjusted to around 7.6 with $NaHCO_3$.

2.2 Analysis of ammonia, nitrite and nitrate

Total ammonia (NH_3 and NH_4^+) contents were determined by the hypochlorite oxidation reaction; Nitrite was measured by the sulfanilamide reaction; Nitrate was analyzed by using UV spectrophotometry [11].

2.3 Isolation, purification and identification of bacteria in the activated sludge

10ml activated sludge was taken from the reactor to study the microbe. The bacteria were isolated by using the standard dilution [12], two kinds of culture mediums were used. One was as follows (g/l): NH_4Cl 1.0, $NaNO_2$ 0.5, glucose1.5, $MgSO_4{\cdot}7H_2O$0.5, $KH_2PO_4$0.7, $CaCl_2$0.3, NaCl0.2, $NaHCO_3$0.5, and agar17. The other culture medium was beef peptone substrate. We identified the main bacteria of the pure cells by the conventional methods and 16Sr RNA method [12–14]. The colony and cell morphology in the sludge were determined by using scanning electron microscopy (SEM, FEI-XL30, Netherlands) and transmission electron microscopy (TEM, FEI-Tecnai, Netherlands).

2.4 Measurement of anammox capability of the seven strains

The changes in concentration of NH_4^+ and NO_2^- in the solution were used to check whether the purified bacteria had anammox activity. The axenic glass reagent bottles (150ml) contained purified cells suspension of 10^7 cell/ml and 50ml axenic solution whose component was same as the influent. After gassing with argon (Ar) replacement of air, the bottles were sealed with Teflon/silicon stoppers. The bottles were put into cultivation box under the condition of 70 rpm and 34℃. Every other day, we sampling from the bottle and measurement the value of ammonium and nitrite, and then sealed stoppers.

3. Results

3.1 Enrichment culture of anammox bacteria

An enrichment culture was eventually prepared by continuously supplying the reactor with the mineral medium containing NH_4Cl and $NaNO_2$ at increasing concentrations (Fig. 1). The population of the sludge changed gradually. Then the autotroph and anaerobe became the dominant population, and the anammox bacteria became main microbe in the sludge instead of other bacteria. After 190 days, the removal efficiencies of NH_4^+–N and NO_2^-–N were both more than 99%, and the volumetric conversion rates of NH_4^+–N and NO_2^-–N were both 99.9 mg/l/d. The main reaction equation was as follow: $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$. The anammox bacteria had been enriched successfully, and had a balanced ecosystem.

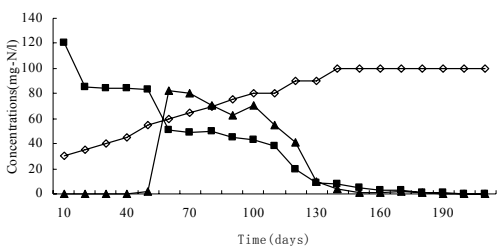

Fig. 1 Changes in concentrations of NH_4^+–N, NO_2^-–N and NO_3^-–N. Influent NH_4^+–N or NO_2^-–N (◇)(The influent concentration of NH_4^+–N and NO_2^-–N was same, so they were the same line); Effluent NH_4^+–N(■); Effluent NO_2^-–N(▲)

3.2 The species of purified bacteria in the anammox sludge

Seven strains were purified by conventional methods. Their numbers are E1–E7. The seven species could grow under limited-oxygen and anaerobic condition. Based on the morphological, physiological, biochemical and 16Sr RNA analysis, these bacteria belonged to *Pseudomonas sp* (E1), *Clostridium beijerinckii* (E2), *Bacillus cereus* (E3), two kinds of *Bacillus* sp. (E4 and E5), *Sphingosinicella microcystinivorans* (E6) and unidentified species (E7). The concentration of these bacteria was very low, except that E1 had a high concentration which was more than 10^7cell/ml; therefore E1 was one of dominant bacteria in the ecosystem. They might play an important role in the anammox reaction.

Fig. 2a shows the shape and size of the bacteria. Most of these bacteria are oval or short-bacilliform. The size is 0.5-0.8×0.8-2.0, the cell is single. The shape was something like the the cells in the community in the anammox sludge (Fig. 2b).

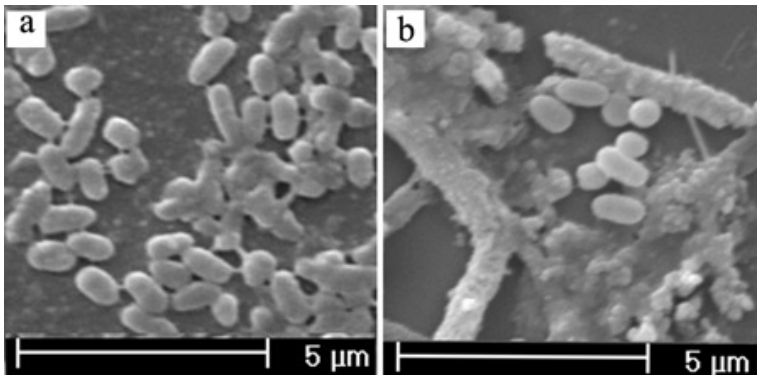

Fig. 2 Scan electron micrographs of bacteria. a. SEM of E1 strain; b. SEM of dominant bacteria in anammox activated sludge.

E1 bacteria could grow under inorganic culture medium and anaerobic condition, and were Gram-negative. The bacteria secreted yellow and green soluble coloring matter. Fig. 3 shows the cell has flagellums at one end.

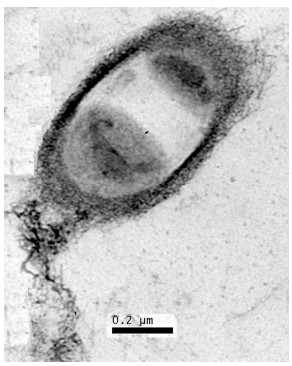

Fig. 3 Transmission electron micrograph of E1 strain.

3.3 Anammox activity of the purified bacteria

Only E1 strain could make ammonia decrease in anaerobic condition, and in the other bottles the concentration of ammonium was almost invariable. That is to say E1 have the anammox activity.

Table 1 shows the anammox activity of E1 strain. The removal efficiencies of NH_4^+–N or NO_2^-–N were 32.14% and 100% respectively. The ratio of removed NH_4^+–N and NO_2^-–N was 1: 3.11, which was higher than the ratio (1: 1.31) of other scientist research [15], and was also higher than the theoretical ratio 1: 1.67 [16]. The result showed that anammox reaction and denitrification reaction existed at the same time, but the capability of denitrification was more than the capability of anammox. Anammox reaction equation was as follow: NO_2^-+ $NH_4^+ \rightarrow N_2+2H_2O$, and denitrification equation was as follow: $6[H]+2\ NO_2^- \rightarrow N_2+2OH^-+2\ H_2O$(or $4NO_2^- +2H_2O \rightarrow 2N_2+4OH^-+3O_2$) .

Table 1 changes in concentration of ammonium and nitrite due to E1 strain.

Time(days)	0	2	4	6	8	10
NH_4^+–N(mg-N/l)	100	97.21	89.47	68.54	65.9	65.9
NO_2^-–N(mg-N/l)	100	94.35	79.9	10.44	0	0

4. Conclusion

Anammox bacteria were successfully enriched in a UASB reactor. The removal efficiencies of NH_4^+–N and NO_2^-–N were both more than 99%. From the anammox sludge, seven species were isolated and purified by conventional methods. The concentration of cells was not high except E1 strain (>10^7cell/ml). E1 have the same shape as that of the colony in the sludge. Its high cells concentration shows E1 strain is one of the dominant bacteria of the anammox ecosystem. From the character and the sequence, we named it *Pseudomonas sp*.

Of the seven species, only E1 bacteria had anammox activity. E1 were denitrifying bacteria, and had a high denitrification capability. Most scientists think anammox bacteria belong to *Planctomyces*, but in this research other genus *Pseudomonas sp*. also has the anammox activity. We can conclude some denitrifying bacteria also had anammox capability in optimal condition. To further clarify the roles of E1 strain, it will necessary to simulate the condition of UASB reactor to observe the changes of nitrogen. It is possible that there are different reactions under the different condition with the same bacteria.

Acknowledgements The supports by the Natural Science Foundation of China (No. 20377013) and by the Natural Science Foundation of South China University of Technology (No. 5050760) are gratefully acknowledged.

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Keratinolytic activity of *Streptomyces* sp isolated of poultry processing plant

G. M. Oliveira[1], M. Cortezi[1], J. Contiero[1,*]

[1] Department of Biochemistry and Microbiology, Biological Sciences Institute, São Paulo University - Unesp, São Paulo, Brazil

[*]Corresponding author: e-mail: jconti@rc.unesp.br

The keratin is not degraded by common enzyme, keratinases have the ability to degrade native keratin and others insoluble enzymes. In the present work was studied keratinase produced by *Streptomyces* sp LMI-1 isolated from industrial plant of poultry processing. The enzyme degraded 87% of feathers after 120 h, it was stimulated by Ba^{2+} and inhibited by Ca^{2+}, Mn^{2+}, EDTA and Hg^{+}. The optimum pH and temperature for the enzyme was 8.5 and 60ºC, respectively. The enzyme was stable after 2 hours at 50ºC. The culture broth analysis by thin layer chromatography showed presence of amino acids serine, methionine, proline, tyrosine and leucine after 72 hours of incubation. The microorganism showed potential for use in industrial process because of higher enzyme production and feathers degradation.

Keywords Keratinase, *Streptomyces* sp, pH, temperature, amino acids.

Introduction

The high consumption of chickens meats generates residues, such as the feathers. An alternative to decrease this pollution is the utilization of feather constitutes that can use as animal feed. The biotechnological processing of feathers for the production of feather meal, in contraposition to chemical processing, present advantages such the preservation of essential amino acids (methionine, lysine, histidine), found in sub-excellent levels (Onifade, *et al*. 1998).

Other potential application ok keratinase include the use in leather industry. This activity involves a series of operations, contributing to the major amount of pollution.

The keratin is an insoluble protein, this stability is caused by a high degree of cross-linkage disulfide and hydrogen bonds and hydrophobic interactions, and therefore the degradation occurs for proteases specify (keratinases). The keratinases (EC 3.4.99.11) belong to the group of hydrolases that are capable of degrading keratin.

The stability of the keratinase is influenced for temperature, pH, and chemical substances. Solvent organic as methanol, ethanol and isopropanol can induce the activity of the enzyme.

In this work, the keratinase produced for new strain *Streptomyces* sp was characterized for potential applications in biotechnological processes.

Materials and Methods

Microorganism identification

Bacteria selected were identified based on morphological and biochemical tests (McFadin 2000), comparing the data with standard species and using an API 20 E (Bio-Meriux). Morphological and physiological characteristics of the isolated bacteria were compared to data from Bergey's of systematic bacteriology.

Microorganism and Inoculun preparation

The bacteria used in this study was the strain *Streptomyces* isolated of poultry processing plant. Two millilitres of the 24 h bacterial suspension ($12x10^6$ cells/mL) grown in a nutrient broth at 35ºC and 150 rpm, were used as the inoculum (2%v/v).

Growth determination

Bacterial growth was monitored by measuring the colony- forming units (CFU mL), as described elsewhere (Silva et al. 2001).

Enzyme production

Experiments was carried out in 500 mL Erlenmeyer flasks containing 100 mL with raw feather and mineral medium as a describe by Letourneau et al (1998) for 120 hours at 30°C. the medium composition was: NH_4Cl (0,5g/L), $CaCl_2$ (0,5g/L), $MgSO_4$ (0,2g/L), K_2HPO_4 (0,3g/L), KH_2PO_4 (0,4g/L), yeast extract (0,2g/L), raw feather (10g/L).The pH was adjust for 7.5. The enzyme free cell was obtained through centrifugation at 10 000*g* for 10 min at 20ºC, and culture supernatants was used as crude enzyme preparations.

Keratinase activity

Keratinase activity was determined using the modified method of Bressolier *et al.* (1999). Keratin azure (Sigma Chemical) was used as a substrate. Samples were incubated with 0,013 g of keratin azure in 800ul a 50 mM Tris HCl buffer (pH 8.5) at 50ºC for 1 h with constant agitation at 100 rpm. The supernatant was spectrophotometrically measured at 595 nm for the release of the azo dye. All assays were done in triplicate. One unit (U) of keratinase was defined as the amount of enzyme causing 0.01 increases in absorbance between sample and control at 595 nm after one hour under the conditions given.

Determination of the protein concentration

Protein concentration was determined by Lowry et al. (1951), methodology.

Determination of the optimal pH for activity

The optimal pH for enzyme activity was determined at 50°C over a pH range from 5 to 12. Maclvaine buffer (pH 5.0-7.0), Tris HCl buffer (pH7.0-9.0) and sodium bicarbonate buffer (pH 9.0-12.0).

Determination of the optimal temperature for activity

To determine the optimal temperature for enzyme keratinase, the reactions was carried out at different temperatures, ranging from 30 to 70ºC. The keratinolytic activity at the 50ºC was taken as 100%. The thermal enzyme stability was determined by incubation at temperature from 40°C to 60°C for 1 hour. The enzyme activity was determined on Tris-HCl buffer (pH 8.5), 50 mM.

Effect of ions and EDTA on keratinase activity

The effect of metal ions was investigated using concentrations of 2 and 10 mM. The enzyme was mixed with different metal concentrations and pre incubated for 15 min at ambient temperature before assay. The control was done with enzyme without metals (100%).

Results and Discussion

Values extremes of pH generally denature the enzymes (Bobbie & Bobbie, 1995). In Figure 1 the optimal pH for enzyme activity was 8,5. This result contrast with the obtained for Bernal et al, 2003 whose optimal pH was between 9.0 and 10.0. Other keratinolitics enzymes showed active at pH alkalophilic, like those secret by Streptomyces albidoflavus (Bressollier et al, 1999).

Initially, with the increase in temperature, the molecular activity was increased, and then the enzymatic complex was increased. The continue increase in temperature can cause a gradative thermal enzyme inactivation, reaching the total thermal enzyme inactivation, because of denaturation by heat. In general the enzymes reacts slowly on the freezer temperatures and the activity increase with increases in temperature, reaching an optimal activity at 45ºC (Bobbie & Bobbie 1995).

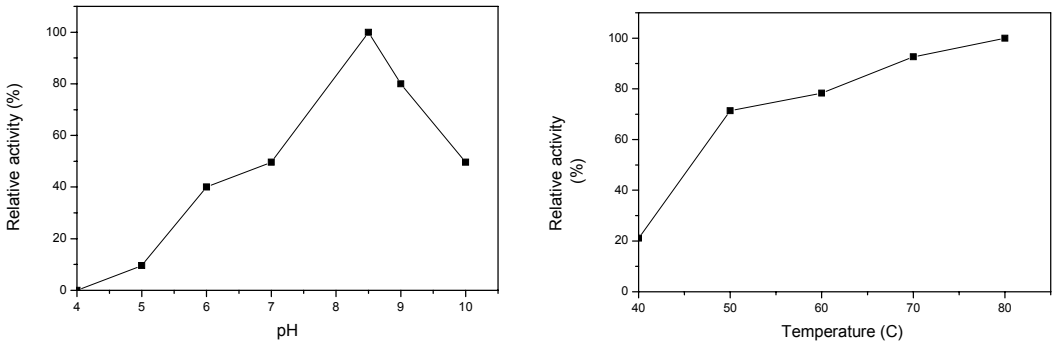

Figure 1: Keratinolitic enzyme activity of Streptomyces sp at different pH and temperature.

The Figure 1 shows the optimal temperature was 70°C. This result contrast with the obtained for Bernal et al, 2003. The optimal temperature enzyme activity obtained by Riffel et al, (2003) was 75ºC, while the optimal temperature obtained by Lin et al (1992) was of 50ºC.

When keratinase enzyme was incubated at 40ºC a light increase on the activity was displayed. At 50ºC, after 2h of incubation a decrease in residual activity occur as a show in Figure 2. When the enzyme was incubated at 60ºC, the relative activity reaches 40% after 3 hours. Figure 2 shows that the enzyme when incubated at 50ºC kept 50% of activity after 7 hours of incubation.

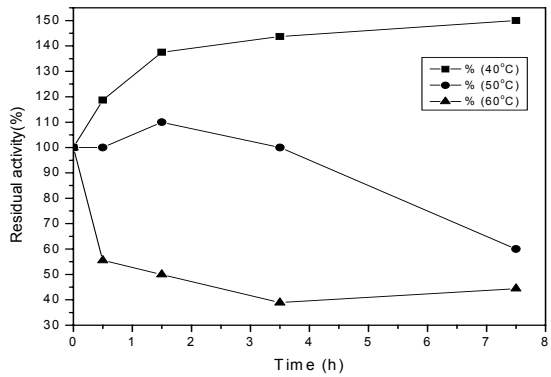

Figure 2: Thermal stability of keratinase produced by *Streptomyces* sp

The enzyme was stimulated by $BaCl_2$ at concentration 2 mM (Table 1), being that the same is not observed in the concentration of 10 mM. This result suggests that the Ba+2 ions are necessary for the enzyme activity and are concentration dependent. In this case, the fact of the ionic ray of the Ba+2 to be the greatest ions metallic used (134pm), suggests that it is most appropriate to set in the active site of the enzyme, helping, of this form, to keep the formation of the complex enzyme - substrate. The enzyme activity was inhibited strong by EDTA, $CaCl_2$, $MnCl_2$, $ZnCl_2$ and HgCl at concentration 10mM. The $MgCl_2$ did not show great influence on the activity. The inhibition for $CaCl_2$, $ZnCl_2$ and EDTA also was observed by Letourneau et al (1998) for keratinase of *Streptomyces* S.K1-02.

The inhibition by EDTA suggests that the enzyme is one metaloprotease, on time that EDTA would be sequestrating some important metallic ion for the keratinase activity. Riffel et al (2003) showed that keratinase was inhibited by EDTA and Cu^{+2} and its activity was increased in the presence of Ca^{+2}. In this exactly work, the researchers affirm that several peptidases are inhibited by the zinc excess, particularly in neutral and alkaline medium. The metallic ion probably acts as a salt or as a species of "bridge" to keep the structure of the enzyme conformation or to stabilize the linking of the substrate on the enzymatic complex. The metallic ion also exerts an important role in the thermal stability of proteases (Suntornsuk & Suntornsuk 2005).

Table 1: Effect of ions, chemical and protease inhibitors on keratinolytic and proteolytic activity of *Streptomyces* sp.

Substance	Ionico ray (pm)*	Relative activities (2mM)	Relative activities (10mM)
$CuSO_4$	72	93,3%	86,7%
$BaCl_2$	134	138,5%	46,1%
$CaCl_2$	99	85,7%	32,1%
HgCl	127	88,5%	53,8%
$MnCl_2$	80	93,7%	12,5%
$ZnCl_2$	74	78,6%	28,6%
$MgCl_2$	66	105,9%	94,1%
EDTA	-	100%	52,9%

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Microbial characterization of Linear Alkylbenzene Sulfonate degradation in fixed bed anaerobic reactor

Lorena Lima de Oliveira[*,1], **Iolanda Cristina Silveira Duarte**[1], and **Maria Bernadete A. Varesche**[1]

[1] Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo, São Carlos-SP, Brazil. Av. Trabalhador Sãocarlense, 400 – Zip Code 13566-590

[*]Corresponding author: e-mail: lorenaloliveira@yahoo.com.br, Phone: +55 16 33738357

The aim of this work was evaluate the linear alkylbenzene sulfonate (LAS) degradation in anaerobic conditions. It was used different inoculum and support media. Four horizontal anaerobic immobilized biomass (HAIB) reactors were used. After 313 operation days, R1 and R2 degraded 35% and 34% of LAS, while R3 and R4, after 344 operation days, degraded 28% and 27%, respectively. The microbial diversity refer to *Bacteria* Domain was evaluated by PCR/DGGE technique. The change may be occurring because of microorganism's selection by surfactant presence. The biomass present at the end of operation was submitted to cloning and DNA sequencing techniques. It was observed the reactors showed large numbers of clones related to Clostridia. Probably, these microorganisms were involved in LAS degradation.

Keywords HAIB reactor; linear alkylbenzene sulfonate; DGGE; 16S rRNA.

1. Introduction

Several microbiologic systems using pure or mixed cultures and different nutritional condition have been used in surfactants degradation. The linear alkylbenzene sulfonate (LAS) degradation involves the breaks of the alkyl chain, the sulfonate group and, finally, the aromatic ring [1].

According Schöberl (1989) [2], the ways to LAS aerobic degradation involve the following steps: (1) conversion oxidative of one or two methyl groups of the alkyl chain to a carboxylic group (ω-/β- oxidation); (2) oxidation of the alkyl chain (β-oxidation); (3) oxidation of the aromatic ring; (4) it breaks of the connection C-S, liberating sulfate (dessulfonation). Probably, the early degradation happens for the ω-oxidation, with following β-oxidation of the alkyl chain, following for opening the aromatic ring and dessulfonation.

The final stage of LAS degradation is the aromatic ring opening. After that, the degradation progresses rapidly with the formation of biomass, carbon dioxide, water and sulfate. The most difficult stage is the breaks of radical alkyl and sulfonate aromatic ring link [3].

The anaerobic microorganisms can use the sulfonate compounds in three ways: a) as electrons acceptors; b) as electrons donors to anaerobic respiration; or c) as substrate to fermentation. The sulfonate group reduction was discovery in pure cultures of *Desulfovibrio desulfuricans*, isolated from marine sediments. This organism was capable of reducing the sulfonates in lactate combination as electrons donor and co-substrate [4].

Denger *et al*. (1996) [5] isolated *Clostridium* sp., capable of use 2-(4-sulfophenil) butyrate as sulfur source. Denger and Cook (1999) [4] used cultures enriched with LAS in anoxic conditions, in medium with glucose. These cultures used the surfactant as sulfur source to microbial growth. The authors obtained the greatest values of similarity to *Aeromonas* (88.2-90.1%) and *Shewanella* (87-88.1%). These microorganisms were described as capable to use LAS sulfur in anaerobic conditions.

Thus, the main aim of this work was compare different inoculum and support media in LAS removal. However, it was intended to identify, by molecular biology techniques, the microorganisms involved in this surfactant degradation.

2. Material and Methods

Four horizontal anaerobic immobilized biomass (HAIB) reactors were used. The description of support media and sludge used as inoculum are showed in Table 1.

Table 1 Reactors characteristics applied in LAS degradation.

Reactors	Support Media	Inoculums
R1	Polyurethane Foam	Anaerobic sludge of UASB reactor used in wastewater plant treatment
R2	Polyurethane Foam	Anaerobic sludge of UASB reactor treating swine wastewater
R3	Charcoal	
R4	Expanded Clay / Polyurethane Foam	

The hydraulic detention time (HDT) used was 12 hours, according to reactors useful volume. Some changes in synthetic substrate composition were made to minimize the extra cellular material production and improving LAS removal efficiency.

2.1 Microbial diversity analysis

The microorganisms identification from reactors R1 and R2 occurred by cloning from total genomic DNA. The products from ten independent PCR reactions were pooled, purified with GFXTM PCR DNA purification kit (GE Healthcare), and concentrated in a speed vacuum concentrator 5301 Eppendorf, A-2-VC rotor. The purified PCR product (50 ng) was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA), according to manufacturer's instructions and transformed into competent Escherichia coli JM109 cells. About 100 clones containing inserts were randomly selected from the library for following sequencing.

The 16S rRNA gene inserts were amplified from plasmid DNA of selected clones using the universal M13 forward and reverse primers. PCR products were checked in 1.2% agarose gels and purified as previously described for following automated sequencing in the MegaBase DNA Analysis System 1000 (GE Healthcare). The sequencing was carried out using the M13, T7 and 782 (Chun 1995) reverse primers and the DYEnamic ET Dye Terminator Cycle Sequencing Kit for automated MegaBase 500 system (GE Healthcare), agree the recommendations of the manufacturer.

The identification was achieved by comparing the contiguous 16S rRNA gene sequences obtained (*500 bp in length) with 16S rRNA sequence data from reference and type strains, as well as environmental clones, available in the public databases Genbank (http://www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project, WI, USA, http://www.cme.msu.edu/RDP/html/index.html), using BLASTn and RDP sequence match routines.

The microbial diversity in reactors R3 and R4 was evaluated by PCR/DGGE technique. The biofilm samples were colleted in different support media. The DNA was extracted according Griffiths [6] method. In these samples, the bands individual identification was made by cloning. The clones from each band were sequencing and they were used to identify the bacteria species present in the bands.

It was used specifics primers to Bacteria Domain [7] to RNAr 16S fragments amplification. Those fragments were separated by Denaturing Gradient Gel Electrophoresis (DGGE).

3. Results and Discussion

3.1 Mass balance

The reactors were monitoring for 313 days (R1 and R2) and 344 days (R3 and R4). The mass balance was calculated after extraction of adsorbed LAS in biofilm (Table 2). R1 and R2 showed better LAS degradation, 35% and 34%, respectively when compared to reactors R3 and R4 that showed 28% and 27% LAS degradation, respectively, at the end of operation. This difference in efficiency may be because of more LAS mass applied in reactors R3 and R4 and because of the support media.

Table 2 LAS mass balance in reactors

Reactor	Period (days)	LAS influent (mg)	LAS effluent (mg)	Adsorbed LAS (mg)	Degradation (%)
R1	286	5782	3616	165	35
R2	286	6008	3771	177	34
R3	314	7854	5461	195	28
R4	314	11066	7221	835	27

3.2 Microbial diversity

A total of 53 clones were obtained in R1. The sequences of inserts were determined and analyzed using Genbank's BLASTn routine. According to analysis, 43 sequences were related to sequences from organisms phylogenetically positioned in the phylum Firmicutes.

For the samples of the reactor R2 were obtained 63 clones of which 51 were related also to the phylum Firmicutes, specifically to the class Clostridia, order Clostridiales. The other 12 clones were related to phylum Proteobacteria and distributed in the following way: 9 related to Alpha-proteobacteria, 2 related to Delta-proteobacteria and only 1 related to Beta-proteobacteria.

The bacteria of the reactor R1 were related to different genera, among them, *Closdridium*. The bacteria of this genus accomplish dissimilative metabolism of sulfate reduction, besides it produce organic acids and alcohols starting from carbohydrates or peptone [8]. In the last phase of operation of the reactor R1, the composition nutritional favored the growth of such cells, once there was carbohydrates readiness, amino acids (yeast extract) and sulfate originating from sulfite oxidation released in breaking the aromatic ring of LAS molecule.

Another clone was related to the *Bacterium* [9] with 97% of similarity. These bacteria were found in systems of activated sludge fed with phenol as carbon source. In reactor R1, the phenol presence was not detected, however, toluene and benzene were observed in close concentrations to 20 μg/L. Probably those compositions were originated from the connection rupture of the aromatic ring with the alkyl chain and they served as organic substrate for the growth of such cells.

A same clone of the phylum Proteobacteria was related to two species of *Beijerinckia*, both with 93% of similarity. Such genera can produce mass highly viscous, semi-transparent in role of the nutritional conditions. Organic substrate as glucose, fructose and sucrose are oxidized to carbon dioxide and acetic acid. The probable growth of such cells in the reactor R1 can be associated to the sucrose presence, which stimulated the formation of polymeric organic in the biofilm.

In the reactor R2, of the 51 clones belonging to the phylum Firmicutes, Class Clostridia, 25 clones were related to *Clostridium*. Many clones presented similarity among 88 to 99% with 25 species different from *Clostridium*. Most of the species of that genus produces ammonia, sulfite of hydrogen, and high amount of H_2. Besides, they fasten atmospheric nitrogen, ferment carbohydrates and produce acetate, butyrate and ethanol.

Most of the clones were related to the species *Clostridium* sp. found in reactor of fluidized bed sulfate reducer used to treat wastewater containing metals [8]. The possible presence of bacteria similar to described them, previously, justify the probable use of LAS as carbon source or other present organic substrate in the biofilm, besides the reduction of sulfate originating from sulfite oxidation.

A clone related to *Azorhizobium caulinodans* (97 to 98%) was observed that to fix N_2 in micro-aerobic conditions. Among the sugars, only the glucose is oxidized. That species grows with propionic, butyric, isobutyric, valeric, isovaleric and caproic acids. Probably, the growth of these cells has been favored by the organic acids produced in all stages.

Only, a clone was included in the class Beta-Proteobacteria. That clone was related to two different species, *Comamonas* sp. (98%) and *Variovorax paradoxis* [10] (96%), bacteria that reduce nitrate and they are capable to degrade phenol.

The class Delta-Proteobacteria gathers the reducer sulfate bacteria. The clones related that class presented similarity with species of the Syntrophobacter (92-95%) [11] and *Syntrophobacter wolinii* (95%) [12]. The growth of these cells is favored in co-culture with *Desulfovibrio*. They oxidize propionic to acetate, H_2 and CO_2. They request the presence of consuming organisms of H_2, which were verified through optical microscopy, acted by fluorescent rods (hidrogenotrophic archaea cells).

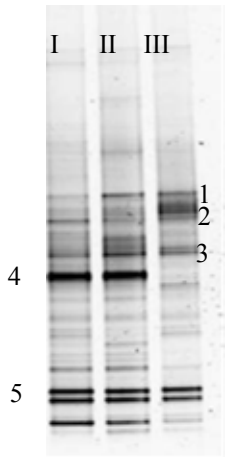

Fig. 1 Gel of DGGE of the samples of the biofilm in the different materials supports of the reactors R3 and R4 using *primers* for Bacteria Domain (I) expanded clay, (II) polyurethane foam and (III) charcoal to the 266 days of operation.

Techniques of molecular biology (PCR/DGGE) were used in removed samples from reactors R3 and R4 after 266 days of operation. Such analyzes allowed to evaluate the present microbial community's structure in each one of the materials support (Figure 1).

The marked bands in Figure 1 were excised and sequenced, being possible to identify the populations of bacteria. In that way the band 1 presented similarity with *Holophaga* (88%); band 2 presented similarity with Delta Proteobacteria (95%); band 3 presented similarity with *Verrucomicrobium* (98%); band 4 presented similarity with Bacteroides (99%) and band 5 presented similarity with Gama-Proteobacteria (97%).

Holophaga was found in all materials support used in reactors R3 and R4. That genus was also present in the samples of reactors R1 and R2. These bacteria are obligated anaerobic and they degrade compounds aromatic to acetate [13].

Bacteroides were only observed in reactor of mixed bed (R4) in both supports (clay expanded and polyurethane foam). The main products of anaerobic breathing are the acetic and isovaleric acids [14]. Probably, the largest concentrations of acetic, isovaleric and propionic acids in R4 was due to the presence of these microorganisms in the biofilm, when compared to R3.

Microorganisms of the class Delta-Proteobacteria were observed in all reactors. That class gathers the sulfate reducing bacteria, among them bacteria of the genera *Desulfovibrio*. They are sulfate reducing bacteria (SRB), strictly anaerobic and they reduce sulfate, sulfite and thiosulfate to H_2S. The main donors of electrons are H_2, lactate, ethanol, malate and fumarate.

4. Conclusion

The LAS mass balance in reactors showed the inoculums were able to degrade surfactant with efficiencies between 27% and 35%.

The analysis of fragments of the 16S rRNA gene showed the reactors had microorganisms strictly anaerobic. The most clones from samples of reactor R1 and R2 was related to *Clostridium*. The bacteria of this genus probably were responsible for LAS degradation.

Beside *Clostridium*, other genus of sulfate reducing bacteria can be related to surfactant degradation since they were identified in all reactor studied. Among these bacteria, it can be on focus the bacteria belonging to Delta-Proteobacteria class, specifically *Desulfovibrio*.

Acknowledgments The support by FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) is gratefully acknowledged.

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Microbial communities from caves with paleolithic paintings

M.C. Portillo[1], J. M. Gonzalez[1], and C. Saiz-Jimenez[1]

[1] INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGIA, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

Recent studies on the microorganisms involved in biodeterioration are revealing the existence of highly diverse and complex microbial communities. The results obtained during our latest studies showed the presence of numerous microorganisms never or rarely reported before in caves with rock art paintings. Among these microbial groups, the presence of sulfate-reducing bacteria (mainly belonging to the Deltaproteobacteria), Bacteroidetes, Chloroflexi, Crenarchaeota, Gemmatimonadetes, Nitrospirae, Planctomycetes, Verrucomicrobia, and several uncultured bacterial candidate divisions have been frequently detected. The metabolic capabilities of the microorganisms that have not been previously cultured are generally unknown, and most of the microbial groups mentioned above have never, or rarely, been cultured. Consequently, there is no information on their potential role on the deterioration of the studied paintings is unknown.

Keywords microbial communities; caves; paleolithic paintings; biodeterioration; microbial diversity

1. Introduction

Although the effect of microorganisms on cultural heritage has been overlooked for a long time. The importance of microorganisms in cultural heritage have been generally overlooked as a consequence of different reasons. One of these causes is that the methods used to detect microorganisms were not adequated to the purpose. Missing significant information on the presence and role of microorganisms presents potential implications in cave conservation. In environmental microbiology, using the appropriated methodology is of critical importance. Classical microbiological methods involved the use of culturing techniques for the detection of microorganisms. Culturing implies a need to grow up a microorganism in the laboratory for it to be detected. Since the growth conditions of most microorganisms in our planet are still unknown, we can only culture up to 1% of the microorganisms present in any given sample [1, 2]. Microscopy observation, for instance, is unable to permit microbial identification due to the relatively low morphological diversity with respect to the known specific diversity of microorganisms.

In recent years, a novel methodology is being developed to approach the detection of environmental microorganisms independently of a need to culture them. The use of molecular techniques based on the analysis of nucleic acids is transforming the way environmental microbiology, and the general microbiology, is being looked up. Today, it is known that the microbial diversity in our planet is huge, well beyond what can be experimentally determined [3]. These molecular methods allow to detect microorganisms without a need for culture them and so the researchers can get a vision of the actual microbial community *in situ*, without modifications due to microbial growth in the laboratory.

Studies available so far report on the isolation and analysis of a very reduced number of microbial groups. For instance, some heterotrophic bacteria and fungi are often cultured and are generally relatively easy to grow up in the laboratory. However, most physiological groups of microorganisms have never, or rarely, being grown up from cultural heritage samples. There are many examples including highly significant microbial groups in most environments. For instance, sulfate reducing bacteria present a potential for causing serious damage to prehistoric paintings [4]. Anaerobic bacteria, because of a higher difficulty in their cultivation have been generally unexplored during culturing surveys. Lithotrophic microbial communities are completedly unknown in caves containing paleolithic paintings. Physiological groups of microorganisms such as denitrifiers, methanotrophs, methanogens, Fe-reducers, S-oxidizers, ammonium-oxidizers, nitrogen-fixers, and a really long list of characteristic microbial metabolisms are often excluded from microbiological studies of cultural heritage. An example of frequently cultured microorganisms are the phototrophs. Microalgae and Cyanobacteria have been reported to grow on historic sites, generally leading to green-colored films able to cover large surfaces of murals, paintings, or walls of a variety of monuments. Some phototrophs able to grow up at reduced light intensities have been often reported to develop in caves with permanent illumination installed [5].

2. Materials and Methods

A case study carried out in Altamira Cave (Cantabria, Spain) is presented here. The methodology used during this study has been previously described [2, 4, 6]. Briefly, DNA and RNA were extracted from minute samples. Analysis based on DNA allow to detect the microorganisms present in a sample, while those performed based on RNA provide with information on those showing significant metabolical activity within their community. Bacterial and archaeal 16S rRNA gene fragments were amplified by PCR (Polymerase Chain Reaction) and processed for DNA library construction with the aim of sequencing and microbial community fingerprints by DGGE (Denaturing Gradient Gel Electrophoresis). Bioinformatic analyses were performed in order to obtain the taxonomic affiliation of the retrieved sequences which was compared with molecular profiles of these communities obtained by DGGE.

3. Results and Discussion

Intensive microbial surveys performed in Alatamira Cave have been aimed to understand the microbiology of this unique environment. One of the objectives of this work was to determine which microorganisms are present, are actively participating in community development, and analyze their role in cave ecosystem, and potential risk for the paintings conservation. While microbial surveys based on the culture of microorganisms resulted in the detection of three single microbial groups: Actinobacteria, Firmicutes, and Proteobacteria [7]. The first survey of Altamira Cave microorganisms based on DNA amplified 16s rRNA gene fragments [8] resulted in the identification of other microbial groups never detected before, such as Acidobacteria, Bacteroidetes, Chloroflexi, and Planctomycetes. In addition, these molecular results appeared to suggest that the Actnobacteria were not the most abundant bacteria in the cave. Further results confirmed this preliminary hypothesis. In fact, the Proteobacteria are by far the most abundant microroganisms in the studied cave. Molecular analysis have also determined that Proteobacteria belonging to the Alphaproteobacteria and Gammaproteobacteria were the most abundant subgroups although the Betaproteobacteria and Deltaproteobacteria represented high fractions of the total microbial community [4, 9]. These results have been complemented with determination of those microroganisms showing significant metabolical activity in these communities. A recent study by Portillo et al. [9], determined that the Alphaproteobacteria and Acidobacteria were among the most active microbial groups in the cave. From these studies, we know today which microorganisms are the most abundant and metabolically active in the cave including in colonizations developing in the cave such as those showing white, yellow, or grey colorations. However, it is important to underline the interest of a highly significant fraction of the microbial communities which remains undiscovered.

A highly significant fraction of the microbial communities from Altamira Cave corresponds to microorganisms from which their metabolism is not known. These microorganisms always represents a significant proportion of the studied communities and often represent more than 30% of these communities although their proportion varies between samples. Besides, an important point is to understand the role or participation of these unknown, or often forgotten, microbial groups in the cave (Table 1). Focusing in this direction, the discovery and diversity analysis of some of these groups have been already reported. Gonzalez et al. [10] showed the presence of a highly diverse community of Archaea, belonging to the Low-temperature Crenarchaeota. The metabolism of this archaeal group is unknown with the exception the single representative cultured, *Nitrosopumilus*. It is an ammonium oxidizer although it is still not clear if every member of the low-temperature Crenarchaeota shares this same metabolism. The case of the Acidobacteria have also been recently reported [11]. Acidobacteria constitute around 20% of microbial communities in Altamira Cave and have been confirmed their presence as metabolically active bacteria [9]. It is an increadible diverse bacterial group in this cave and the Acidobacteria includes bacteria with a very diverse metabolism ranging from Fe-reducers, to acidophiles, aerobic and anaerobic representatives, and even phototrophic species. All this diverse metabolisms have just been reported from the study of the only five species described so far within this bacterial group. The role of Acidobacteria in Altamira Cave remains unknown. Other groups have been detected and similar comments could be reported for each single one of them (Table 1).

Table 1 Some of the microbial groups rarely or never reported before as important components of the microbial communities in Altamira Cave. Estimate of their diversity, current knowledge of their status, and the level of knowledge on their metabolism are reported.

Microbial group	Status of detection	Abundance	Physiology and role
Sulfate-reducing bacteria	Active/mostly uncultured	Very high	Known
Low-temperature Crenarchaeota	Active/uncultured	Very high	Mostly unknown
Acidobacteria	Active/uncultured	Very high	Barely known and highly diverse
Bacteroidetes	Active/uncultured	Medium/high	Mostly unknown and highly diverse
Planctomycetes	Active/uncultured	Medium/high	Mostly unknown and highly diverse
Chloroflexi	Uncultured	Medium	Mostly unknown
Verrucomicrobia	Active/uncultured	Medium	Mostly unknown
Nitrospirae	Uncultured	Medium	Mostly known

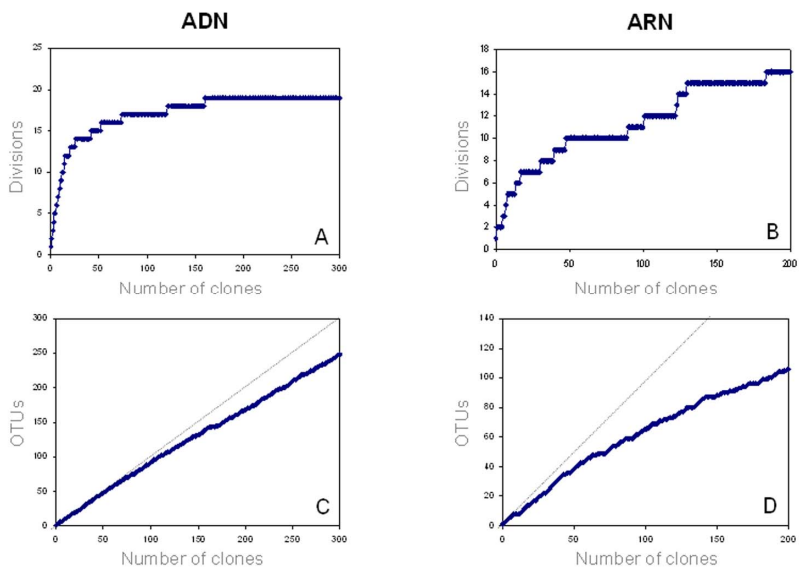

Fig. 1 Cummulative curves of the number of processed sequences retrieved from Altamira Cave and the number of bacterial divisions (A and B) or different microorganisms (shown as OTUs or Operational Taxonomic Units) (C and D) analyzed in base to DNA (A and C) and RNA (B and D).

Many of the detected microbial groups are unknown, and many microroganisms present in Altamira Cave have not been even detected yet. We have performed an analysis of the microbial diversity present in Altamira Cave and observed that the cummulative curves relating the number of processed 16S rRNA gene sequences with the number of distinct microorganisms (or OTUs, Operational Taxonomic Units) show that these curves reach a plateau (Fig. 1) when the bacterial phylla present in the cave are considered both in the case of analysis based on DNA (microorganisms present in the cave) and RNA (microroganisms metabolically active in that environment). However, when this analysis is performed for the number of different microroganisms detected in the cave, the curves barely show an inclination from the diagonal (1:1) straight line for DNA-based analysis (Fig. 1). This result implies that the actual number of different microroganisms in the cave is much higher that the number of already detected microbes. In fact, based on current estimates [3] the expected number could be reported to be around 30,000 microorganisms per gram. In the case of metabolically active microroganisms from a RNA-based molecular survey, the result is similar although the cummulative curve shows a marked inclination towards reaching a plateau and so, we could suggest that the number of microorganisms metabolically active in Altamira Cave is not too far from those already detected in our recent studies. These data

confirm that the microbiology of paleolithic paintings-containing caves is highly complex and the understanding of the interactions, diversity and function of the microorganisms in these caves requires not only further studies, but also the development of novel methodologies that will contribute to solve current gaps in environmental microbiology.

As a consequence of these recent discoveries, likely the best alternatively for cave conservation is the one proposed by some authors [12, 13] suggesting that the best way to preserve prehistoric paintings in caves is to leave them alone. The reason is very simple, we do not know the possible consequences of altering the microbial communities in these subterranean environments.

4. Conclusion

The study of microbial communities in Altamira Cave have revealed the presence of metabolically active microorganisms rarely mentioned in previous studies. Examples are the Crenarchaeota (Archaea), the Acidobacteria and several other bacterial groups (Table 1). These groups represent a significant fraction of these communities with the negative aspect that most of their metabolisms are, at present, unknow. The fact that these metabolisms remain unknown and these microbial communities are barely understood together with the high microbial diversity in this cave represent serious risks for the conservation of this cultural heritage site.

Acknowledgements The authors acknowledge support through a contract with the Ministry of Culture (Contract 31321000710T, "Analysis of the conditions for the conservation of paleolithic paintings in Altamira Cave") and projects CGL2006-11561/BTE, CGL2006-07424/BOS, from the Spanish Ministry of Education and Science (MEC).

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Microbial communities in different volcanic environments from Canary Islands (Spain)

M. C. Portillo[1], S. Sanchez-Moral[2], J. Lario[3], V. Soler[4], and J. M. Gonzalez[1]

[1] INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGIA, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain
[2] MUSEO NACIONAL DE CIENCIAS NATURALES, CSIC, Jose Gutierrez Abascal 2, 28006 Madrid, Spain
[3] UNIVERSIDAD DE CASTILLA-LA MANCHA. 45071 Toledo, Spain
[4] INSTITUTO DE PRODUCTOS NATURALES, CSIC, Avda. Astrofisico Francisco Sanchez 3, 38206 La Laguna, Spain

The microbial communities of different sites located within the Canary Islands were studied in relationship to the characterisitcs of volcanic environments. In this study, we focused on the microorganisms thriving in temperate terrestrial environments, specifically boreholes and water mining activities at these islands. Molecular methods were used to detect the presence of microorganisms in water samples. Two boreholes and water mines located in La Palma Island were studied. Results showed the presence of diverse microbial communities which might have significant consequences for the geochemistry of these underground water sites.

Keywords biogeochemistry; microbial communities; volcanic environments; boreholes; water mining

1. Introduction

Microbial communities are the major factor contributing to biogeochemical transformations in our planet. Recent methodological developments are making possible the assessment of natural communities mainly applying novel, culture-independent, molecular techniques based on nucleic acid (both DNA and RNA) analysis [1, 2]. In spite of the possibility of detecting most microorganisms by using these methods, current studies are demonstrating that microbial diversity in most natural environments is so large that is experimentally undeterminable [3]. As a consequence, microbial community surveys aim to detect those microorganisms representing the most common fraction of these communities and the metabolically active microorganisms which participate directly in ongoing biogeochemical processes in the environment under study [2, 4]. However, most of the diversity existing in a given sample will remain unknown due to methodological constrains.

The study of microbial communities is a first, and necessary, step to get a general understanding on micro- and macro-biotic systems and their interactions with their geochemical surroundings. As an example, finding and utilizing natural water sources represents an activity of general interest. For instance, human communities inhabiting places with scarce water at the land surface have historically dedicated a significant effort to search and explote any potential source of drinking water, being underground water the most commonly origin of the tap water supplies in most populations. The development of microbial communities in underground water systems directly influence water quality and the geochemistry of the underground system. Understanding these sites and the interaction among biological and abiotic components of these locations will contribute to the management and conservation of these valuable water sources.

In this study, the microbial communities of selected low temperature water systems from Canary Islands (Spain) were studied. Molecular methods were used to detect the major components of the microbial communities at these sites which composition was characterized.

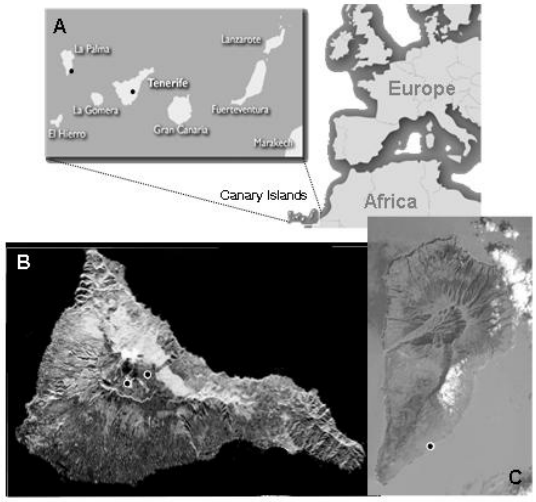

Fig. 1 Location of Canary Islands, off the northwestern african coast (A) and a detail of Tenerife Island (B) and La Palma Island (C) with indication of the sampling sites (circles).

2. Materials and Methods

Canary Islands are of volcanic origin and signs of volcanism are still detected in these islands. A number of systems are newly formed or destroyed depending on volcanic activities currently occurring at these islands. Water supplies in these islands is a serious concern for people living there. Search for water sources in the islands attracts significant funding from regional budgets. In this study, we analyzed samples obtained from two of the islands Tenerife and La Palma (Fig. 1). At Tenerife Island, samples were collected from boreholes around the Teide submit. These boreholes allow the access of scientists to natural underground water reservoirs and have been previously described in detail [5]. Interestingly, these reservoirs, located below 350-400 m depth, showed an unexpected high CO_2 concentration (around 25-30% of air volume) which is detected from about 50-100 m above the water level [5]. Experimental data were collected at two boreholes (Fig. 1) named El Portillo (hereafter EP) and Montaña Majúa (MM), percussion-drilled by the Water Authority of Tenerife (Consejo Insular de Aguas) for piezometric studies and water quality control. MM is located in the central area of the Caldera, 2264 m above sea level, reaching a depth of 505 m, well below the water level (at a depth of 446 m), with a temperature of around 13ºC, while EP is located in the northern area of the Caldera, 7.1 kilometres from the first borehole, 2133 m above sea level and it reaches a depth of 400 m (water level is at a depth of 370 m), and presented a temperature of around 11ºC.

A second sampled area was located in La Palma Island (Fig. 1). It constituted some water deposits found in an area of water mining activity focused on finding a water source named Fuensanta, which was covered by active volcanic activity that occurred during a recent eruption at the Teneguia Volcano in 1971 [6, 7]. Water temperature was 30ºC.

The major components of the microbial communities were analyzed by molecular methods based on DNA and RNA. DNA will provide with information on the microorganisms present in the analyzed samples while using RNA, those microorganisms showing significant metabolical activity within the community will be detected. The methods used have been previously described [2, 8, 9] and DNA and RNA extraction, PCR amplification of 16S rRNA gene fragments, cloning, clone screening, sequencing, and sequence data analysis have been performed as reported therein. Microbial communities fingerprinting was performed as described by Gonzalez and Saiz-Jimenez [1] by Denaturing Gradient Gel electrophoresis (DGGE) and were used to characterize the major microbial components of the studied communities. Microorganisms identified through 16S rRNA gene fragment sequencing were located at the migration level in DGGE profiles.

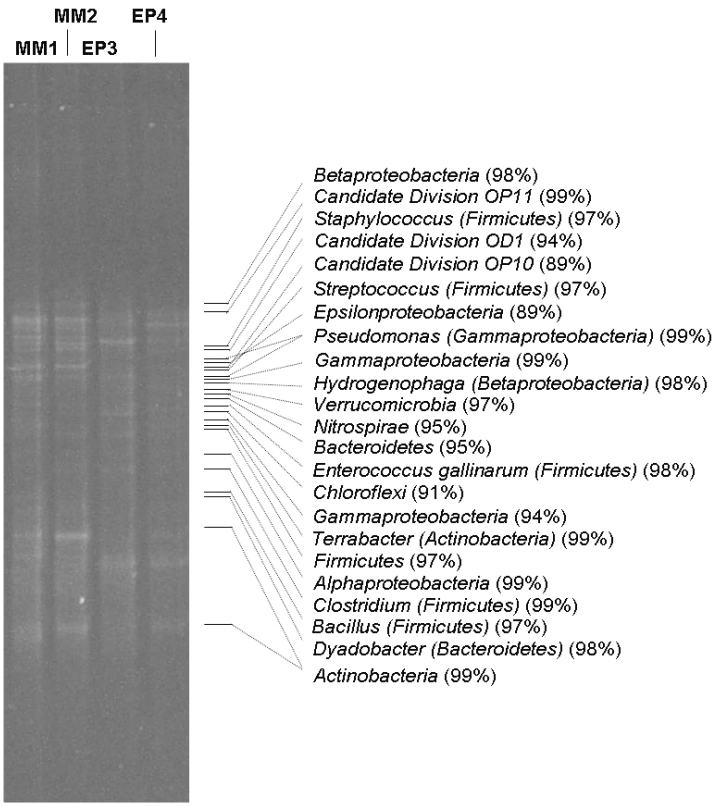

Fig. 2 Microbial community fingerprints and identified bacteria at the two studied boreholes (MM and EP). Two samples from each site were carried out in parallel. These analyses were performed from DNA extracted directly from the collected samples.

3. Results and Discussion

Water is a scarce resource in Canary Islands and current efforts are being carried out to search for new water sources and preserve the ones currently under use. One of the necessary steps towards the understanding of these water reservoirs and their conservation is to comprehend the microbial communities thriving at these aquatic systems in order to deduce their role in these systems.

The analyzed waters contain highly diverse microbial communities composed by a large number of different microorganisms. Replicate analyses of samples collected at these boreholes revealed high reproducibility among replicates and suggest that each one of these two sites could be characterized by their specific microbial fingerprint. At the studied boreholes, the most frequently detected bacterial group is the Firmicutes which representatives related to the genera *Clostridium*, *Bacillus*, *Streptococcus*, *Staphylococcus*, and *Enterococcus*. These members of the Firmicutes generally present a heterotrophic metabolism, being aerobes or facultative anaerobes. The Gammaproteobacteria were the second group represented in the borehole samples. Some of the Gammaproteobacteria detected in these boreholes were related to the genus *Pseudomonas*, which generally shows an aerobic and heterotrophic metabolism. Actinobacteria were also well represented and sequences related to the genus *Terrabacter* were found within this group. Alphaproteobacteria (represented by the genus *Hydrogenophaga* among others) and Bacteroidetes were two bacterial phyla often detected in these samples. Other phyla such as the Chloroflexi, Nitrospirae, Verrucomicrobia, Epsilonproteobacteria and candidate divisions OP10 and OD1 were detected in water collected from the bottom of the boreholes MM and EP. These groups add a complex metabolic diversity to a microbial community mainly based on heterotrophic bacteria.

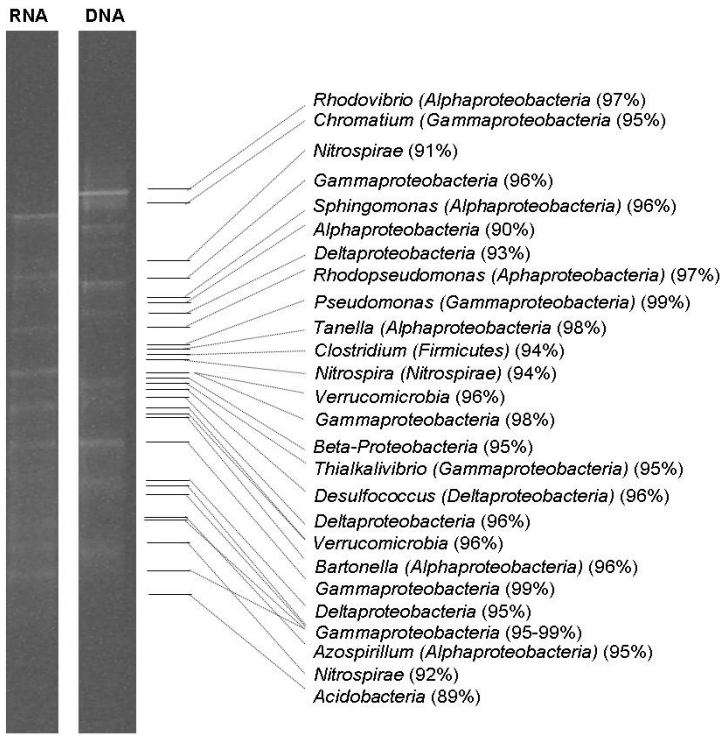

Fig. 3 Microbial community fingerprints and identified bacteria at a water sample collected from water mining at La Palma Island. Samples were analyzed by PCR-DGGE from DNA and RNA obtaining the present and the metabolically active fractions of the microbial communitiy, respectively.

Water collected from the water mining activity beeing carried out at La Palma Island revealed the presence of a diverse microbial community in those waters and a different microbial community structure when analyzed the fraction of microorganisms metabolicaly active and participating directly in the biogeochemical transformations occurring at the collected samples (Fig. 3). A comparison of the profiles obtained by PCR-DGGE based on DNA and RNA showed that the metabolically active microorganisms in that community are not necessarily those showing more abundance as detected by a DNA-based survey of the microbial community. As a consequence, there is a number of microorganisms that are present and even representing an abundant fraction of the community but they are just waiting for better conditions to arrive and being able to replace and outcompete other bacteria in that system. Thus, transformations or alterations of current underground conditions might lead to serious changes in the microbial community structure and so result in potential undesired effects of water quality at these sites.

Among the bacterial groups detected in water collected at the mining site, the most frequently encountered as metabolically active microorganisms corresponded to the Alphaproteobacteria and Gammaproteobacteria which were represented by microorganisms related to the genera *Azospirillum*, *Bartonella*, *Rhodovibrio*, *Rhodopseudomonas*, *Sphingomonas*, and *Tanella* within the Alphaproteobacteria, and *Chromatium*, *Pseudomonas*, and *Thioalkalivibrio* within the Gammaproteobacteria. In these samples, there was a high abundance of metabolically active members of the Deltaproteobacteria and the phylum Nitrospirae. The detected Deltaproteobacteria were classified among the sulfate-reducing bacteria, represented, for instance by the genus *Desulfococcus*, suggesting the existence of sulfate reduction and likely the presence of anaerobic or microaerophilic microenvironments in these waters. The phyla Betaproteobacteria and Verrucomicrobia were also detectable members of the microbial community showing significant metabolic activity in these water mining samples. Acidobacteria and Firmicutes (genus *Clostridium*) were two phyla detected through molecular analysis based on DNA so they were present in the sample but did not represent a significant activity with respect to the total community metabolism. Among these microorganisms there are some directly implicated in the sulfur (*Thioalkalivibrio, Desulfococcus*) and nitrogen (*Azospirillum*) cycles, typical sulfate-reducing bacteria (*Desulfococcus* and other Deltaproteobacteria), or heterotrophic bacteria usually implicated in degrading complex organic substances (*Sphingomonas*, Verrucomicrobia, Bacteroidetes).

For both locations studied in this work, some of the detected bacteria were relatively distant phylogenetically (from 89 to 99% sequence similarity of the 16S rRNA gene) with respect to cultured and described species and some of them have never been cultured and physiologically studied which makes difficult to guess their

potential biogeochemical role in these systems. The gap between phylogeny and metabolism or physiology is an aspect still to be resolved (Gonzalez and Saiz-Jimenez, 2004). The description of novel species, and current efforts in the culturing of microorganisms might be the necessary complement to microbial community surveys using molecular methods due to the biases involved in culturing microorganisms from natural environments [10].

4. Conclusions

Based on the molecular analyses performed during this study, we were able to determine the existence of complex bacterial communities in clearly different underground waters collected from boreholes (at Tenerife Island) and water mines (at La Palma Island). The influence of these communities in the biogechemical cycles and transformations going on at these underground environments are complex and require further studies focusing on specific aspects of these processes. Herein, the presence of metabolically active diverse microorganisms within these communities has been detected.

Acknowledgements The authors acknowledge financing from the Junta de Andalucía to group BIO288.

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Microbial diversity in *Chromobacterium violaceum* determined by 16S rRNA gene analysis

M.L.R. Bastos Silva[1], M.C.C. Pereira Lyra[2], J. Paula Oliveira[2], H. Almeida Burity[2] and G.M Campos-Takaki[3,4,*]

[1]Doutorado em Ciências Biológicas, UFPE, Recife-PE; [2]Laboratório de Genoma, IPA, Recife-PE; [3]Departamento de Química; [4]Núcleo de Pesquisas em Ciências Ambientais – Universidade Católica de Pernambuco, 50.050-900 Recife – PE, Brasil.

[*]Corresponding author: e-mail: takaki@unicap.br, Phone: +55 81 21194017

The aim of this study is focused in the evaluation of the genetic diversity of *C. violaceum* isolated from Brazilian ecosystems. Strains from Malaysia, Amazonas, Pernambuco, Ceará were analysed by the 16S rRNA gene (amplified 16S ribosomal DNA restriction analysis) to define their phylogenetic positions. All strains were grown overnight in LB medium at 30°C at 150 rpm, and cooled on ice, and the DNA was amplified in a thermocycler. The primers used were fD1 (AGAGTTTGATCCTGGCTC AG) and rD1 (AAGGAGGTGATCCAGCC) complementary to the ends of the 16S rDNA. The data obtained herein demonstrated that this method allowed grouping the *C. violaceum* isolates according to different Brazil States. However, the distances genetics between among all the isolated ones studied in this work demonstrated low variability but that the use of 16S rDNA gene tree revealed the good correlation between phylogenetic clustering and geographic origin.

Keywords Phylogeny, 16S rRNA, distances genetics

Introduction

Chromobacterium violaceum is a versatile Gram-negative, classified in the Betaproteobacteria class, order Neisseriales, family Neisseriaceae [1]. This free-living bacterium presents a high flexibility to survivein the most diverse environments [2]. This bacterium is a common inhabitant of soil and water confined to tropical and subtropical regions. The most notable characteristic of *C. violaceum* is the production of the chemically well-characterized pigment named violacein [3].

The use of the polymerase chain reaction (PCR), automated DNA sequencing has led to a greater understanding of bacterial taxonomy [4, 5]. In spite of these advances, it is widely held among microbiologists that most bacterial species have been neither isolated nor characterized [6 - 7]. The use of rRNA analysis alone has shown that there is much greater diversity among bacteria than was previously suspected [8]. The application of advanced molecular techniques to environmental microbiology has greatly facilitated the identification and characterization of new bacterial species. The coupling of molecular analysis methods with bacterial systematics and phylogenetic analysis software has made it much less difficult to establish relationships between species [9-11].

One of the primary tools used in bacterial identification and phylogeny is rRNA sequencing and analysis [12, 13]. Ribosomal RNA is almost perfectly suited for characterization of prokaryotes because of its functional consistency [13,14]. There are three known rRNA molecules found in bacteria. These are the 5S, 16S, and the 23S rRNAs, composed of approximately 120, 1600, and 3000 nucleotides respectively [15, 16]. Of these three, the 16S rRNA has proven to be the most beneficial in bacterial identification [17, 11]. Sequencing of the 16S rRNA gene usually requires genomic DNA extraction, amplification of the 16S rRNA gene using universal bacterial primers and PCR, and an automated DNA sequencer [13]. The DNA sequence obtained by this method must be referenced against a known set of sequences in a database. In this study we evaluate the genetic diversity of isolated *Chromobacterium violaceum*.

Materials and Methods

***Bacterial strains*-** The 14 strains used in this study are collection the NPCIAMB – UNICAP (Núcleo de Pesquisas em Ciências Ambientais – Recife – Pernambuco – Brazil) included type strain ATCC 12472 was isolated from soil in Malaysia the entirely genome executed by the Brazilian National Genome Sequencing Consortium.

Extraction of genomic DNA- Genomic bacterial DNA was extracted as described by [18] Extracted DNA was resuspended in TE buffer plus 10 mg/ml of RNAse and its integrity after extraction was determined using 0.8% agarose gels in TE buffer as described by [19].

16S ribosomal RNA gene amplification- The complete 16S rRNA gene was amplified by PCR using the primers fD1 AGAGTTTGATCCTGGCTC AG) and rD1 (AAGGAGGTGATCCAGCC) [10]. Polymerase chain reaction mixtures (50 µl) consisted of 0.2 mM of each dNTP, 10 pmol/µl of each primer, 1 U of Taq DNA polymerase (Invitrogen, Brazil), and 50 ng of bacterial DNA. The thermal cycling conditions consisted in one cycle at 94°C for 3 min followed by 30 cycles of 50 s of denaturation at 92°C, 50 s of annealing at 57°C, and 1 min of extension at 72°C, and a final extension step of 7 min at 72°C. Direct sequencing of the PCR products purificated with the high pure PCR Product Purification Kit (Roche). The PCR products were visualized after electrophoresis a 100 Volts during 1h 30 min in agarose gel, SYBR Gold Nucleic Acid Gel Stain and photographed under UV light.

16S ribosomal RNA gene sequence analysis- 16S ribosomal RNA gene sequence analysis- Sequencing was Macrogen Advancing through Genomics in Korea. Phylogenetic analysis was inferred by BioEdit is a biological sequence alignment editor version 7.0.9.0. Mega 4.0 Program [20] (Molecular Evolucionary genetics Analisys) used for Phylogeny Test was one thousand bootstrap resembling were used to evaluate robustness of the inferred trees, Neighbor Joining Method, Model for nucleotide calculated by Tamura-Nei. Additional 16S rRNA gene sequences of *C. violaceum*, *Neisseria gonorrhoeae* were obtained from GenBank Database. *N. gonorrhoeae* were used as out group.

Results and Discussion

The DNAs of isolates were subjected to the PCR reaction with primers specific to region 16S rRNA and resulted in the amplification of a fragment of approximately 1200pb. The molecule of smaller subunit of 16S rRNA was established as a universal marker, and added the techniques of DNA sequencing and analysis of software more accessible emerged a taxonomy based on DNA, and unthinkable to publish a description of a new species and for the establishment its correct taxonomic position [21].

The sequences of isolates were subjected to BLAST specializing in NCBI (BLAST/bl2seq) to verify the identities of the pair, now confirmed as of *C.violaceum* showed 95-99% of identity with the strain of *C.violaceum* UCP1489 isolated in the state of Pernambuco (Fig. 1). The identity is the result of the alignment between two similar sequences that will have a stamp rolling from 75% of homology [22].

The isolated UCP1467 of the Amazon region and collected by the Federal University of Amazonia (UFAM) showed the greatest distance gene between pairs of fourteen isolates analyzed with an average of 0.36% of divergence in relation to others. All other pairs had a distance between 0.0, 0.01, 0.02 to 0.03% with a total of 856 sites analyzed. Showing that the comparative analysis of the primary structure of the genes of 16S rRNA turned the microbial taxonomy in a simpler system for the identification of systematic based on these sequences and their evolutionary histories [15]. Since, at the time, only approximately 1 to 10% of microorganisms can be isolated by method of culture, in the liquid or solid [23, 24] and relatively low rates of new microorganisms have been characterized and currently cultivated [25].

The presence of the strain of *Neisseria gonorrhorae* (NC_002946) as a crew generates questions on bootstrap confidence of the largest group, with 10 isolates (Fig. 2) plus their training is highly reliable for the subgroup formed by isolated from *C. violaceum* UCP1035, UCP1466 and UCP1489 and the crew composed of UCP1467. In alignment with *N. gonorrhorae* was obtained a total of 490 sites kept between 1581 generated (36%) and between the alignments conducted exclusively with isolated from *C. violaceum* the number of sites has been kept from 1001/1482 (67%) showing that even with the divergence the isolated UCP1467 and rich ecosystems of their region of origin analyzed the data are not sufficient to report a new species. Moreover, Hungria et al [26] analyzing sequences of the gene 16S rRNA checked the genetic diversity among isolates collected by the UFAM in Rio Negro - Brazilian Amazon and presented by the diversity that the isolated Brazilian could included in two new groups of species of *C. violaceum* already described.

Conclusion

The broad genetic diversity of ecosystems tropical and subtropical can justify continental and regional variations in different strains of *C. violaceum*, when observed variations of sequences of 16S rRNA of up to 95-99% of identity.

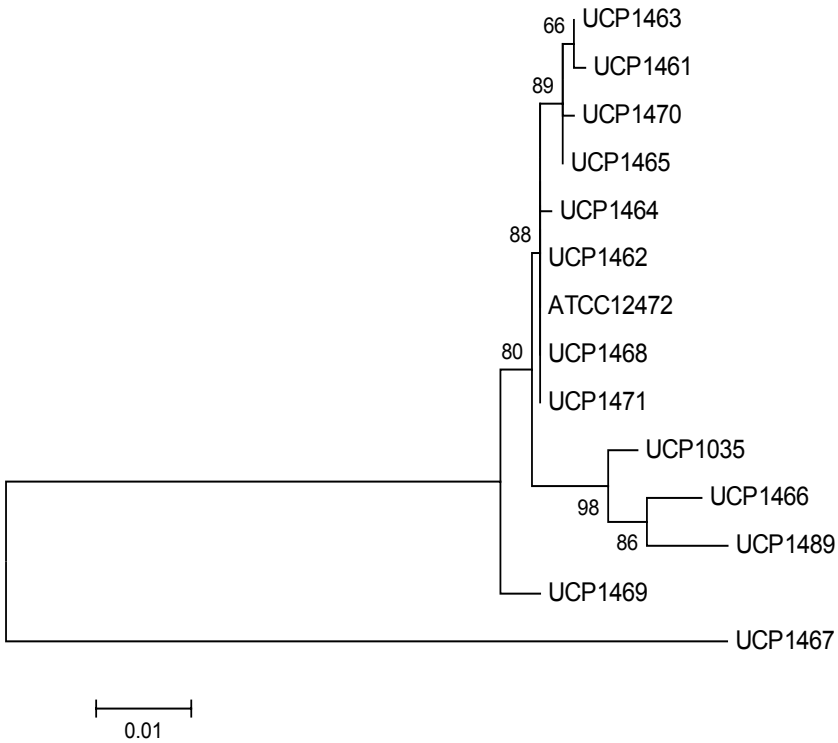

Fig. 1: Phylogenetic tree based on 16S rDNA partial sequences of *C. violaceum*: UCP1461, UCP1462, UCP1463, UCP1464, UCP1465, UCP1466, UCP1467, UCP-1468, UCP1469, UCP1470, UCP1471 (Amazon), UCP1489 (Pernambuco) and UCP1035 (Ceara) States of Brazil and compared of *C. violaceum* ATCC 12472 (Malaysia), obtained by the method of Neighbor-Joining with bootstrap 500 and model of Tamura-Nei (Mega4).

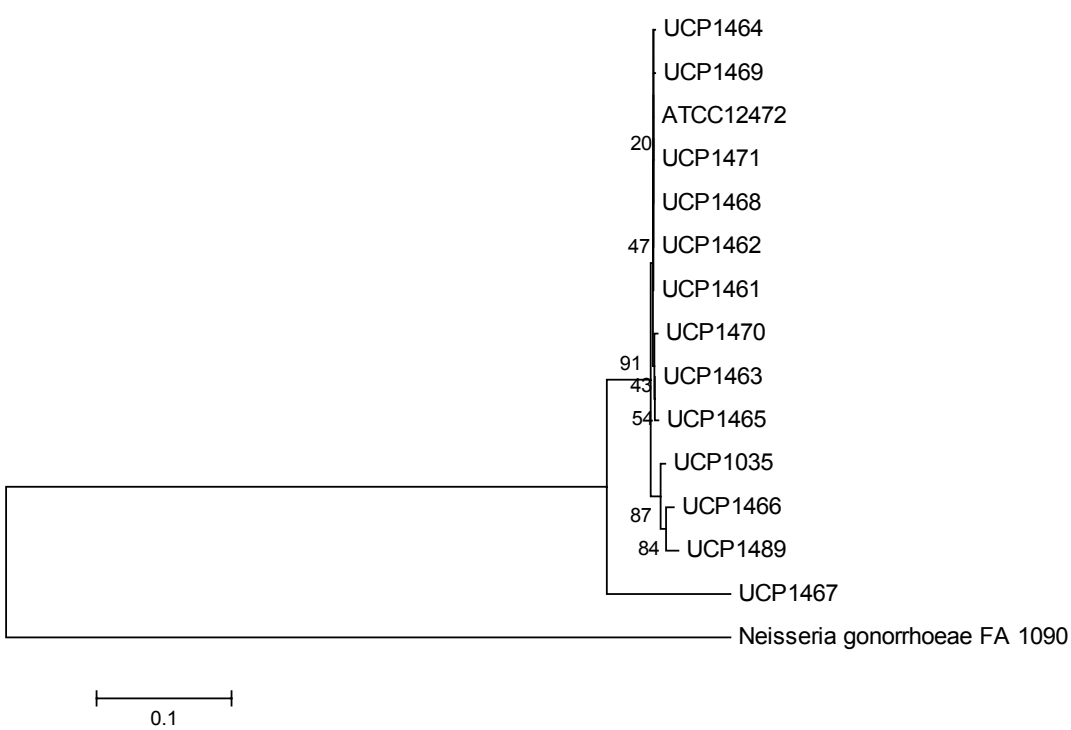

Fig. 2: Phylogenetic tree based on 16S rDNA partial sequences of *C. violaceum*: UCP1461, UCP1462, UCP1463, UCP1464, UCP1465, UCP1466, UCP1467, UCP-1468, UCP1469, UCP1470, UCP1471 (Amazon), UCP1489 (Pernambuco) and UCP1035 (Ceará) States of Brazil and compared of *C. violaceum* ATCC 12472 (Malaysia) with the crew *Neisseria gonorrhorae*, obtained by the method of Neighbor-Joining with bootstrap 500 and model of Tamura-Nei (Mega4).

Acknowledgements We acknowledge the financial support given by CAPES (Brazil) in the form of a fellowship to M.L.R.B Silva. This work was supported by CNPq, UNICAP (Brazil) and IPA (Brazil).

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Microbial Pandora's box : Interactions of free living protozoa with human pathogenic bacteria

A. Martín-González[1,*], M.T. García[2], C. Pelaz[2], and J.C. Gutiérrez[1]

[1] Dpto. Microbiología-III, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain
[2] Laboratorio de Legionella, Centro Nacional de Microbiología, Inst. de Salud Carlos III, 28220 Madrid, Spain

[*]Corresponding author: e-mail: anamarti@bio.ucm.es Phone: +34 913944968 Fax: +34 913944964

Protozoa seem to be the main reservoirs of important and common pathogenic bacteria. The existence of these microbial Pandora's boxes might explain the survival and persistence of bacterial pathogens in diverse natural and artificial habitats. We describe the peculiar characteristics of these eukaryotic microorganisms which can explain their role as pathogen source. Besides, we have reviewed in some detail the diversity and incidence of bacteria-protozoa interactions that can be evolved from bacterial mechanisms to avoid predation and specially digestion by protozoa. Biological and sanitary consequences from human pathogenic bacteria and free-living protozoan interactions are analyzed. Future prospects are proposed in order to prevent and control potential risks for humans.

Keywords amoebae; ciliated protozoa; pathogenic bacteria; microbial reservoir; endosymbionts.

1. Introduction: Why protozoa?

Free-living protozoa are eukaryotic microorganisms with ubiquitous distribution in very diverse types of both, aquatic and terrestrial habitats where they contribute significantly to the carbon and nitrogen recycling. Besides flagellates, two main groups of bacterivorous protozoa can be distinguished; amoebae and ciliates which present quite different morphological and physiological features [1]. Amoebae are phagotrophic protozoa, which move by pseudopodia with diverse morphologies, are among the most common and abundant eukaryotic microorganisms in all types of soil habitats [2]. Like amoebae, the vegetative cells of ciliated protozoa have not cell wall , but they show different feedings types and movement mechanism. In natural environment, the nutrition of ciliates mainly depends of the uptake of solid food. Ciliates have cilia distributed in functional specialized groups involved in cellular movement and the predation of bacteria by filter-feeding [1]. Under unfavourable environmental conditions, the vegetative cells of some species of both, ciliates and amoebae can differentiate to resting cysts. In this ametabolic stage, the dehydrated cell is surrounded by a cyst wall with 1-4 layers of diverse chemical composition, which is very resistant to disinfectants, antibiotics and other antimicrobials.

Consumption by protozoa is considered to a major source of bacterial mortality in environment. Selective grazing by phagotrophic protists has been identified as an important shaping force for bacterial evolution. We have reviewed the sanitary importance and the incidence in pathogen evolution of interactions between free-living protozoa and human pathogenic bacteria. Some sanitary and ecological consequences of these interactions are discussed.

2. Results and discussion

2.1 Interactions types between bacteria and protozoa

The types of interactions established between human pathogenic bacteria (and phylogenetically related genera) and free-living protozoa are quite diverse, although some of them have been forced experimentally by co-culture bioassays, what do not indicate they were true in natural environment.

Table 1 illustrates some relevant examples of important human pathogenic bacteria and phagotrophic protozoa, as the amoebae *Acanthamoeba polyphaga* and *Hartmanella vermiformis* and some species of the ciliate genus *Tetrahymena*, well-known microorganisms in basic research. Three different fates of internalized bacteria were reported in co-culture studies: 1) Those bacteria that can multiply and cause the protozoan lysis in advanced stages, e.g. *Legionella pneumophil* and *Listeria monocytogenes* [3-6], 2) The bacteria that can multiply in protozoan cytoplasm but they are unable to produce the reservoir lysis, as *Coxiella burnetti* [7,9] and finally, the bacteria that show digestional resistance but the intracellular replication doubtful and cannot cause the cellular lysis, as for instance, *Burkholderia cepacia* and certain species of *Vibrio* and

Mycobacterium [4,8,9]. It must be pointed out that, detailed molecular studies of most of the interactions should be made in order to determinate clearly the intracellular replication of bacteria in the protozoan and both, the similarities and differences in the development, fate and gene expression of pathogen in this reservoir, in comparison with those of bacteria-mammalian cells models.

Table 1. Main representative respiratory and gastrointestinal human pathogenic bacteria that can use protozoa as reservoirs, at least in co-culture experiments. * = Bacteria-protozoan interactions that have been also detected in natural or artificial habitats.

BACTERIA	PROTOZOAN GROUP/S	INTERACTION TYPE	REFERENCE
Mycobacterium spp.*	amoebae	survival, replication?	[8,9]
Vibrio	amoebae	survival, replication?	[4]
Salmonella	amoebae/ciliates	intracellular replication	[11]
Shigella sonnei	amoebae	survival, replication?	[12]
Campylobacter spp.*	amoebae/ciliates	survival	[8]
Listeria monocytogenes	amoebae	intracellular replication	[5,12]
Helicobacter pylori	amoebae	replication at low temperature	[8,13]
Burkholderia cepacia	amoebae	survival, intracellular replication?	[6]
Escherichia coli 0157/others	amoebae/ciliates	survival	[14]
Francisella tularensis	ciliates	intracellular replication	[5]
Chlamydia spp.	amoebae	survival, spore formation	[9]
Legionella spp.*	amoebae/ciliates	intracellular replication	[3,4]
Staphylococcus aureus MRSA	amoebae	intracellular replication	[15]
Chlamydophila pneumoniae	amoebae	intracellular replication	[9]

By other hand, the application of molecular methodologies have shown that both, the nuclei and cytoplasm of some amoebae and ciliated protozoa contain certain genera bacteria, related with genera of bacterial human pathogens, specially of obligate and /or facultative intracellular bacteria. This second group of bacteria interacting with free-living protozoa are able to maintain a steady relationship with their hosts and apparently they do not thrive outside them, therefore these bacteria are considered endosymbionts. So, different evolutionary lineages of bacterial endosymbionts of acanthamoebae have been identified to date, which are found within the *Alphaproteobacteria*, the *Betaproteobacteria*, the *Bacterioidetes* and the *Chlamydiae* [16]. In ciliates, intracellular bacteria are mainly restricted to the macronucleus of the host, as *Holospora obtusa*, macronuclear endocytobiont of *Paramecium tetraurelia*. A very interesting relation in that between the intracellular bacteria of the genus *Caedibacter* and the freshwater ciliate *Paramecium*. *Caedibacter* endocytobionts can infect the ciliate *Paramecium*, but also kill potential uninfected hosts before an infection and reproduction of bacteria can occur. This contradiction is due to a toxic and complex protein produced by the bacteria in a non reproductive phase of their life cycle. Paramecia continuously release endocytobionts, whereby both forms, the vegetative growing form and the toxic non-reproductive form. The lethal effect of the toxic form depends on the presence or absence of *Caedibacter* inside a receiver *Paramecium.* Therefore, the toxic bacteria, after inclusion into a food vacuole, cause the death of all those paramecia that do not possess *Caedibacter* cells, or that harbour another *Caedibacter* species. Feeding rates and reproduction of the predators *Didinium nasutum* (ciliate) and *Amoeba proteus* (naked amoeba) were not influenced by whether or not their paramecia prey were infected [17].

It is commonly accepted that the origin of these interactions are bacterial post-ingestional adaptations to protozoan grazing, once inside the food vacuole, bacteria have developed strategies to resists acidification and enzymatic degradation. Like in mammalian cells, several facultative and obligate intracellular pathogens can survive and replicate successfully inside protozoan cells [18]. These interactions can persist along the time in environment and, consequently, both types of microorganisms (prokaryotic and eukaryotic) become co-evolve when the interaction is specific and mature.

2.2. Biological and ecological consequences / traits from these interactions

They are very diverse and have important ecological and sanitary consequences, such as those stated in the following points:

1) The associations between pathogens and protozoa seem to be a major reason to explain the presence and persistence of bacteria in environment. Bacterial internalization provide a protective effect against unfavourable environmental conditions, including predation and starvation. For instance, *Legionella pneumophila* inside protozoa is more resistant to biocides, high temperature, acidity and high osmolarity [3]. Coliforms and other bacterial pathogens have increased resistance to free chlorine when ingested by ciliates or amoebae. Furthermore, it is proven in co-culture bioassays that protozoa release membranous vesicles, containing aggregates of viable pathogenic bacteria, since they cannot digest them [3,11]. Vesicle membrane and /or the presence of aggregates may have shielded the bacteria from different types of stresses and the presence of effective concentrations of biocides.

2) It have been demonstrated that after internalization in protozoan hosts, the pathogenic bacteria are more infectious and virulent for mammalian cells and organisms [3,4,8]. The causes of this fact are still unknown, it should be make a detailed study of bacterial gene expression, specially of those genes involved in pathogenesis, when prokaryotes are inside protozoan reservoirs in comparison with the gene expression pattern in mammals.

3) A remarkable number of the most common protozoan species (amoebae, ciliates) can encyst under stress conditions. Some studies have reported that bacterial pathogens can remain long time inside these resting cysts which can resist extreme conditions [5,9]. It is probably that under these circumstances bacteria enter in viable non culturable stage. Within resting cysts , pathogens are undetectable by methodologies based in culture and they cannot be eradicated by the usual control procedures.

4) Experiments with different serovars of *Salmonella enterica* and protozoa from different habitats indicate that bacteria are grazed at different rates depending on the O-antigen variability so, at least amoebae can recognise O-antigens with different efficiencies, i.e., their receptors have different affinities for the distinct O-antigen epitopes [19]. Unlike amoebae, ciliates , which are filter-feeders, do not recognise their preys by cell-to-cell contact buy filter prey by size.

5) At least in *Legionella pneumophila*, co-culture with protozoa can resuscitate the bacterial cells in viable non-cultivable stage after water disinfection with sodium hypochlorite . The intracellular bacteria inside trophozoites of *Acanthamoeba polyphaga* block encystment and the resistance of both microorganisms to disinfectant is enhanced [20].

3. Conclusions and future prospects

Co-culture experiments denote that interactions of free-living protozoa with human pathogenic bacteria suppose a potential sanitary risk that could generate important incidences in Public Health. In our opinion, international authorities should promote cooperative international researches to evaluate the real risks of these interactions and to prevent them. Some previous studies should be made in order to achieve the mentioned general purposes, such as the followings:

***** Exhaustive quantitative and qualitative analysis in diverse natural and artificial habitats to know the real occurrence of these interactions between free-living protozoa and human pathogenic bacteria.

***** Detailed molecular studies of each interaction to determinate accurately the fate of each bacterial species and if protozoa can be used as alternative experimental models to mammalian cells.

***** Detection of interactions between bacteria and other phagotrophic free living protozoa as heterotrophic flagellates.

***** Development of fast and accurate methods to detect the presence of pathogens within both, vegetative cells and resting cysts of protozoa.

* An evaluation of the antibacterial efficiency of biocides with diverse chemical nature to destroy more common human pathogens, specially those involved in waterborne diseases, when they are inside protozoa.

Results from these studies will provide important data to make sanitary adequate legislations to prevent and control human diseases acquired by this route.

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Microbiological resistance of optical sights for civilian and military use

E. Ståhl Wernersson[*,1] and H. Håkanson[1,2]

[1] Environmental Laboratory, GS Development AB, Jägershillgatan 15, SE-213 75 Malmö, Sweden
[2] Department of Biotechnology, University of Lund, Box 124, SE-221 00 Lund, Sweden

[*]Corresponding author: e-mail: eva.stahl.wernersson@gs-development.com Phone: +46 40 671 50 00

Civilian and military equipments are used at various places all over the world which requires that the design of the equipment needs to withstand different climatic zones. High and low temperatures can be tested in hot cabinet and freezers. Humidity cycles can be tested in environmental chambers. The microbiological resistance of equipment needs a more advanced procedure. Red dot sights for civilian and military use were inoculated with a mixture of fungi. The test procedure is based on a Military Standard. The functionality and the condition of the sights were checked before and after the test. Five species of fungus *Aspergillus niger*, *Aspergillus flavus, Aspergillus versicolor*, *Pencillium funiculosum*, and *Chaetomium globosum* were pre-cultured. A mixed spore suspension with a total concentration of $1 \cdot 10^6$ spores/ml was blended in equal parts. The sights were sprayed with the mixed spore suspension and incubated at 30 °C, relative humidity 95 %, for 30 days in an environmental cabinet. The viability of the mixed spore suspension was checked. The sights passed the functionality test after the fungus test period.

Keywords fungi; material test; environmental test; aluminium; electro; optical; mechanical; equipment;

1. Introduction

Mechanical, electrical, optical equipments are used at various places all over the world. This requires that the equipment needs to be designed to withstand different climatic zones. Normally the equipment is used in the temperate zone; however there are extreme zones like very hot deserts (+60 °C) and arctic zones with temperatures down to -55 °C. The humidity may vary from < 10 % up to 100 % relative humidity (tropical rain forest). Furthermore the temperature may change rapidly over a wide range within a 24 hours cycle.

The equipment should not only withstand temperature changes and high relative humidity, but also microbes that might be found in these areas. To protect the equipment some kind of enclosures is used which can be made of materials such as plastic, metal, glass or a combination of them. However most equipment has a machine to man interface such as knobs, displays, contacts or lenses. The interface surface between the sealed box and contacts and/or adjustments is a risk for contamination by microbes and humidity. High and low temperatures can be tested in hot cabinet and freezers. Humidity cycles can be tested in environmental chambers. The microbiological resistance of equipment needs a different procedure.

We have chosen red dot sights as an example of an equipment used in a vide range of environmental conditions. The sight is a sealed unit with optical lenses consisting mainly of aluminium, brass and glass. The intensity of the red dot is manually set by a switch. The sights were inoculated with a mixture of five different species of fungi. The test procedure is based on a Military Standard [1]. The functionality and the condition of the sights were checked before and after the fungus test.

2. Material and Methods

2.1 Sights

The type of sights used in the tests was CompM2 (Aimpoint AB, Malmö, Sweden). It consists of a black anodized aluminium tube with a battery chamber, see Figure1. The battery is a lithium battery (DL1/3N), also used in cameras. The light source is a specially designed light emitted diode with extremely low energy consumption. The front lens has a special coating which transmits all visible wave lengths except for a small range in the red area. The diode is placed in focus of the front lens and the observer sees the reflecting image of the diode. The intensity can be regulated from very low currents, nA, up to mA, to cover the needs in various light conditions. The sight is sealed by o-rings and adhesive to make it water proof.

Fig. 1 The sight with flip covers. The tests were made without flip covers.

2.2 Cultivation of fungi

Five species of fungus *Aspergillus niger* (ATCC9642), *Aspergillus flavus* (ATCC9643), *Aspergillus versicolor* (ATCC11730), *Pencillium funiculosum* (ATCC11797) and *Chaetomium globosum* (ATCC6205) were purchased from CCUG [2]. Each fungus was cultivated separately on potato dextrose agar (Merck 1.10130.0500) or mineral salts agar according to Table 1.

Table 1 Fungus and corresponding media.

Fungi	USDA	ATCC	Medium (pure culture)
Aspergillus niger	QM 386	ATCC 9642	Potato dextrose agar
Aspergillus flavus	QM 380	ATCC 9643	Potato dextrose agar
Aspergillus versicolor	QM 432	ATCC 11730	Potato dextrose agar
Penicillium funiculosum	QM 474	ATCC 11797	Potato dextrose agar
Chaetomium globosum	QM 459	ATCC 6205	Mineral salts agar

A mineral salts solution was prepared by dissolving KH_2PO_4 0.7 g, K_2HPO_4 0.7 g, $MgSO_4 \cdot 7H_2O$ 0.7g, NH_4NO_3 1.0 g, NaCl 0.005 g, $FeSO_4 \cdot 7H_2O$ 0.002 g, $ZnSO_4$ $7H_2O \cdot 0.001$ g and $MnSO_4 \cdot H_2O$ 0.001 g, in 1000 ml of osmotic water**.** The pH was adjusted between 6.0 and 6.5, using a calibrated pH meter PHM210 and a pHC2015-8 electrode (Radiometer Copenhagen, Denmark). 15.0 g of agar (Oxoid, Basingstoke, Hampshire, England) was dissolved in a liter of the mineral salts solution. The solution was kept refrigerated at 6 ± 4 °C.

The fungi were individually incubated at 30 °C on agar plates until fruiting bodies were developed. Ten ml of osmotic water containing a wetting agent (0.05 g/l), sodium dioctyl sulfosuccinate (SDS), was poured to each cultured fungus. The surface growth was gently scraped from the culture with a rounded glass rod. The harvest was put into a 125 ml Erlenmayer flask, containing 45 ml water and 50 to 75 solid glass beads, diameter 5mm. The procedure was repeated 3-5 times. The E-flask was vigorously shaken and the solution was filtered into a flask, using a funnel with a layer of glass wool. The filtered spore suspension was centrifuged (BB3V, Jouan, S.A., St Herblein, France) and the residue was washed in 50 ml of osmotic water. The filtered solution was centrifuged again and the washing procedure was repeated further two times. The final residue was dissolved in 20 ml of mineral salts solution. The total procedure was conducted for each species of fungus.

The number of spores for each culture was counted using a Neubauer improved counting chamber and a microscope (XSP-136A, Sagitta, Mariestad, Sweden). The spore suspensions were diluted to contain $1 \cdot 10^6$ spores/ml. A mixed spore suspension with a total concentration of $1 \cdot 10^6$ spores/ml was blended in equally parts.

2.3 Viability check

Each spore suspension, 0.2-0.3ml, was inoculated on individual agar plates according to Table 1. The plates were incubated at 24°C to 31°C for 7 to 10 days and the fungal growth was visually checked.

A test solution was prepared for the viability test of fungus used in the test chamber. Glycerol 10.0 g, KH_2PO_4 0.l g, NH_4NO_3 0.l g, $MgSO_4 \cdot 7H_2O$ 0.025 g, yeast extract 0.05 g and SDS 0.005 g were dissolved in 100 ml osmotic water. The pH was adjusted to 5.3. Cotton strips were dipped in the solution and the strips were dried before inoculation of test pieces in the test chamber. The dried strips were placed vertically close to the test items so that the test strips and test items experienced the same test environment. According to the military standard the length of the strips shall be at least the height of the tested sights but not in contact with each other.

2.4 Test performance

The five sights were visually inspected and the functionality was checked. In the functional test the intensity of the different settings of the switch is checked. The lowest settings are observed with a night vision device (NVD) since the intensity is so low that the light can not be seen with the naked eye.

Before inoculation the sights were kept at 25 °C and 95 % relative humidity for four hours. The five sights were inoculated with the mixed spore suspension until drops began to form on the surface. The cotton strips were also inoculated. The sights and the cotton strips were incubated at 30 ± 2 °C and 95 % relative humidity. The temperature was checked with a calibrated Pt100 (ETI Precision s/n D9811393, Pentronic, Gunnebo, Sweden). After 30 days the sights and the cotton strips were removed from the test chamber. The growth of fungi was evaluated and the function of the sights was checked.

2.5 Other environmental tests

The five sights were also tested regarding storage, temperature shock and function at extreme temperatures, as well as humidity. In the storage test, the sights were cycled four times between -55 °C (Laboratory freezer, SE215, TEFCOLD A/S, Viborg, Denmark) and +71 °C (Drying oven, TS8000, TERMAKS, Bergen, Norway) and kept at corresponding temperature for at least 8 hours. Functionality was tested at the end of the last cycle at +71 °C and at the end of the last cycle at -55 °C. In the temperature shock test, the sights were kept at -45 °C for at least 4 hours and then moved to the hot cabinet +71 °C within one minute. In the humidity test the sights were kept in an environmental chamber (type KBP6395F, TERMAKS, Bergen, Norway) at 95 % relative humidity and the temperature was cycled between 30 °C and 60 °C, for 5 days. The temperature and the humidity were logged using Testo177-H1 (Nordtec Industri AB, Göteborg, Sweden) in the environmental chamber. The temperature in the freezer and the hot cabinet was registered using a logging system (ACC-2[16], INTAB, Stenkulla, Sweden) with thermocouples (K).

3. Results

Pre-cultivation of the fungi resulted in spore concentrations according to Table 2. The number of spores was counted twice and the average value is shown.

Table 2 Concentration of spore solutions after pre-cultivation of fungi.

Fungi	10^6 Spores/ml
Aspergillus niger	17 ± 1
Aspergillus flavus	13 ± 1
Aspergillus versicolor	6.4 ± 0.4
Penicillium funiculosum	10.4± 0.6
Chaetomium globosum	1.1± 0.2

Chaetomium globosum is a slow-growing organism which used 18 days to develop fruiting bodies, compared to 7 days for the other types of fungi. All fungal spore suspensions were viable and the viability of each separate fungus on the agar plates is shown in Figure 2. The agar plate with *Chaetomium globosum* is covered with thin green filamentous growth (difficult to see in the picture).

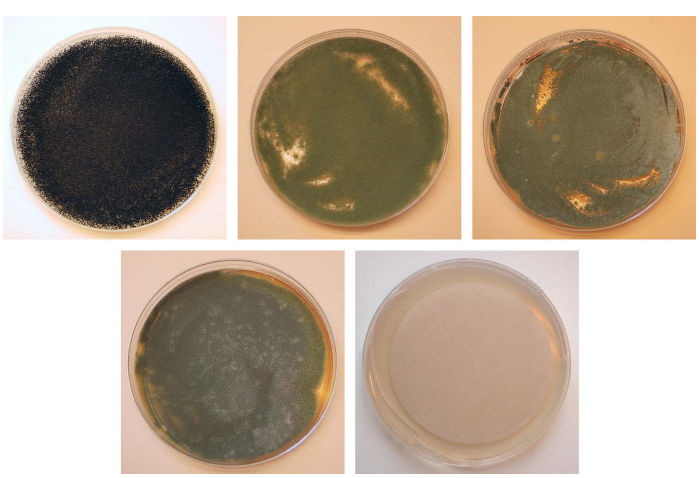

Fig. 2 Viability check of *Aspergillus niger* (upper left), *Aspergillus flavus* (upper middle), *Aspergillus versicolor* (upper right), *Penicillium funiculosum* (lower left), *Chaetomium globosum* (lower right).

The spore suspensions were diluted and counted before mixing the spore solution for all five fungi, resulting in a total volume of 25 ml. In the test chamber, see Figure 3a, the viability was checked after 7 days and growth was detected for > 90 % of the area on all cotton strips. After 30 days the growth of fungi had increased on the cotton strips, as shown by the intense colour Figure 3b.

Fig. 3a (left) Test chamber at the end of test.
Fig. 3b (right) Cotton strip after 30 days of incubation.

There were traces of growth from organic substances, rather scattered and spares on the surfaces of the five sights, Figure 4a, 4b and 4c.

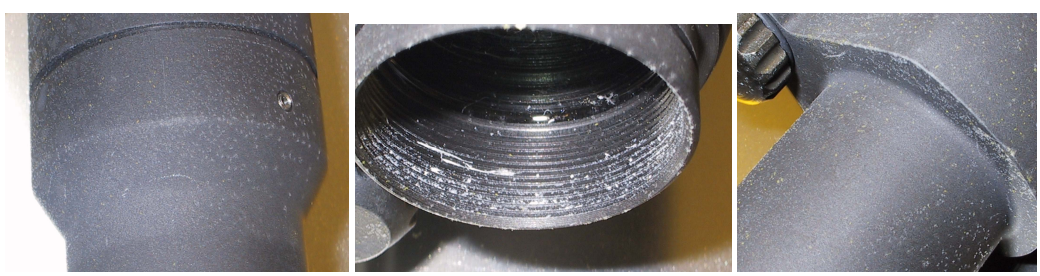

Fig. 4a, 4b and 4c Fungal growth on the sights at evaluation.

The surfaces of the sights were not affected by any deterioration and it was possible to remove all traces of fungal growth by washing the sights with water solution containing a mild detergent. The lenses showed no fungal growth on the surfaces, nor were the sealing of the sights affected by fungal growth. All sights passed the functionality test after the fungus test period. According to the visible evaluation table the sights achieved grade 1, see Table 3.

Table 3 Visible evaluation of test item after the incubation.

Amount of growth	Grade	Organic Substance
None	0	No microbial growth
Trace	1	Scattered, sparse or very restricted growth
Light	2	Intermittent infestations or loosely spread microbial colonies on substrate surface Includes continuous filament growth extending over the entire surface, but the underlying surfaces are still visible
Medium	3	Substantial amount of growth. Substrate may exhibit visible structural changes.
Heavy	4	Massive microbial growth. Substrate decomposed or rapidly deteriorating.

The sights were functional after the storage, temperature shock and humidity test and the visual appearance of the surfaces were not altered.

4. Discussion

The most important issue is the function of the equipment exposed to a variety of microbes. The used fungus mix covers a wide range of the common fungus types in the environment [3]. *A. niger*, *A. flavus* and *Chaetomium globosum* have been found among different types of paper and cardboard throughout the seasonal changes for a year in India [4]. Materials commonly found in home; cloth (cotton 65 % polyester 35 %), plywood, carpet (nylon and polypropylene) has been inoculated with *A. niger* and *Chaetomium globosum* to find out different regimes of washing procedure to reduce the spore and mycotoxin levels [5]. *Chaetomium* speices have been isolated from air and surfaces samples in nearly half of the buildings with sick building syndrome [6]. However *Asperigillus* and *Penicillium* species were more common in the number of buildings, 82 % and 92 % respectively. Some of the species are rather dangerous and may cause infections with great influence on the personal involved.

Even in ultra clean environments, like space crafts and the clean room, where those are built, microbes survive [7]. The surrounding environment was 22 °C and the relative humidity 40 %. It was found that these microorganisms were surviving on for example black anodized aluminium, which is the same material as used in the sights. The possibility for fungus to grow on aluminium has been used to study aluminium-colonisation by *A. niger* [7].

We have selected a way to check the function by performing an all over functional test including the most demanding conditions which the device is exposed to. This test includes storage and usage at high and low temperatures. The critical points in equipments are the mechanical interface into the sealed unit, in this case the switch and the battery compartment. The solution for the battery compartment is to seal it with silicon (Loctite, CAF2) and for the switch the compartment is sealed with an o-ring made of neoprene. Our results show no influence of the fungi on either the o-ring or the silicon sealing. Furthermore the tests show that even with extreme temperatures no functional failure was detected.

The sight is a sealed compartment and in the manufacturing process the lenses are glued and checked for leakage. It is important to select the components in such a way that the extreme environmental conditions can be met. This means that the materials; glass, metal, plastic and glue must be seriously evaluated. During production samples are taken arbitrary for tests in an external laboratory to check that the sight fulfils the requirements of the specifications.

This fungus tests were performed for 30 days but the long-term exposure must be evaluated further on. This procedure can be used for other types of equipment when functionality and deterioration due to microbes needs to be checked for.

Acknowledgements Aimpoint AB is gratefully acknowledged for supporting the sights for tests and for communication.

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Molecular and phylogenetic analysis on bacterial strains isolated from a PAHs wastewater treatment plant

Domenico Davolos[*,1], and Biancamaria Pietrangeli[1]

[1] Department of Environmental Health Sciences (DIPIA), National Institute of Occupational Safety and Prevention (ISPESL), Via Urbana, 167 - 00184 Rome, Italy

[*]Corresponding author: Email: domenico.davolos@ispesl.it; Phone: +39064714260. Fax: +39064744017

In this contribution we summarize results of molecular and phylogenetic analysis conducted on hydrocarbon degrading bacteria sampled from a polycyclic aromatic hydrocarbons (PAHs) wastewater treatment plant in Italy. The bacterial strains showed high 16S rRNA gene sequence identity to described taxa from the GenBank (NCBI) belonging to the *Achromobacter*, *Acinetobacter*, *Bacillus*, *Cellulosimicrobium*, *Gordonia* and *Pseudomonas* genera. Moreover, catabolic genes for the hydrocarbon metabolism were investigated through PCR amplification and DNA sequencing. Finally, for the *Achromobacter* sp. strain LU6 and *Pseudomonas* sp. strains LU7 and LU9 phylogenetic inferences were obtained on the genes encoding NOx reductases of the denitrification pathway. The obtained molecular data are addressed to design oligonucleotides for the simultaneous detection by DNA microarray screening of specific genes in hydrocarbon-degrading bacteria.

Keywords Achromobacter; Acinetobacter; Bacillus; Cellulosimicrobium; denitrifying bacteria; Gordonia; Pseudomonas.

1. Introduction

In the context both of industrial wastewaters biotreatment and of bioremediation of contaminated soils, there is interest in isolating bacterial strains harbouring oxygenases and other enzymes with substrate specificities [1]. For this reason, we are characterising bacteria from a number of different samples mainly from industrial wastewaters. Here we used culture-dependent approaches and molecular techniques to study the composition and the genetic diversity of hydrocarbon degrading bacteria isolated from a PAHs wastewater treatment plant in Italy. Enrichment isolation of taxa contributing to the biodegradation processes in this complex system has been successfully applied. The 16S rRNA gene sequences of the obtained isolates were phylogenetically affiliated to the genera *Acinetobacter* and *Pseudomonas* (γ-Proteobacteria), *Achromobacter* (β-Proteobacteria), *Bacillus* (Firmicutes), *Cellulosimicrobium* and *Gordonia* (Actinobacteria). We determined the taxonomic characteristics of these bacterial strains that can grow on hydrocarbon compounds. Finally, because anaerobic oxidation pathways can be of particular importance in oxidized (e.g. denitrifying) redox zones and different denitrifying bacteria using hydrocarbons are promising for application for the bioremediation technologies of petroleum-contaminated sites, molecular and phylogenetic analyses were carried out on the genes encoding NOx reductases of the denitrification pathway [2].

2. Material and Methods

The bacterial strains were isolated on minimal medium agar plates containing diesel as sole source of carbon. The morphology of the strains was determined following staining and examined via phase-contrast and confocal scanning laser microscopy. The 16S rDNA and two transcription factor genes (rpoD and Rho, respectively) of the isolates have been PCR-amplified and sequenced. For the 16S rDNA gene, PCR mediated reaction was performed using universal primers, while the primers used for the genes encoding rpoD and Rho were specifically designed (available on request). Fragments of the genes encoding subunits of the denitrification pathway as well as for the oxygenases of the hydrocarbon metabolism were produced with new specific primers (available on request). Purified PCR products were sequenced on both strands using the same PCR primers. Cycle-sequencing reactions were sequenced on an Applied Biosystems ABI Prism 3100 DNA Sequencer. The obtained nucleotides and the inferred amino acid residues were compared with accessible data in GenBank databases. Phylogenetic analyses were conducted using MEGA v. 3.1 (www.megasoftware.net/) by the Neighbor-Joining (NJ) and Maximum Parsimony methods; in all the phylogenetic reconstruction bootstrap replicates (1,000) were calculated.

3. Results and Discussion

The bacterial strains were closely related (98%-99% 16S rDNA sequence identity) to described taxa from the GenBank (NCBI) belonging to *Achromobacter*, *Acinetobacter*, *Bacillus*, *Cellulosimicrobium*, *Gordonia* and

Pseudomonas genera. The morphology of the strains is shown in the BiomicroWorld2007 virtual Presentations website (http://www.formatex.org/biomicroworld2007/virtual/). For inferring the evolutionary relationships, multiple sequences alignment and phylogenetic trees were obtained by using several methods (Fig. 1). Bacteria inhabiting PAHs wastewater treatment plant are potential agents for hydrocarbon bioremediation, and several papers reported the isolation and characterization of strains belonging to the genera *Acinetobacter*, *Pseudomona*s, *Alcaligenes*, *Gordonia* and *Bacillus* with the ability to grow using PAHs as sole carbon and energy sources [3]. It has been suggested that groups of γ- and β-Proteobacteria and Gram + bacteria play dominant roles in the degradation of oil and petroleum hydrocarbon pollution. A microbial consortium (that included *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*) isolated from a PAHs contaminated land farm site can degrade and mineralize different concentrations of anthracene, phenanthrene and pyrene [4]. Bacterial strains recently isolated from solid waste oil samples that showed capacity of growing in the presence of hydrocarbons were identified by the 16S rDNA gene and belonged to *Bacillus pumilus*, *B. subtilis*, *Micrococcus luteus*, *Alcaligenes faecalis* and *Enterobacter* sp. [5].

It has been established that heterogeneous communities are required to degrade the mix of hydrocarbons (aliphatic hydrocarbons (*n*-alkanes and branched isoalkanes) and the PAHs) present in most oil-contaminated environments. Nevertheless, little is known about the individual functional roles of bacteria within an oil-degrading community. This study has identified key bacterial taxa involved in hydrocarbon degradation but did not determine their respective roles in degrading different petroleum hydrocarbon components because only growth of the above-mentioned strains in mineral media amended with diesel as sole carbon source was carried out. However, many previously characterized petroleum degraders exhibited restricted substrate profiles, e.g. *Alcanivorax* and *Marinobacter* showed a preference for aliphatic hydrocarbons and the *alkSB1GHJ* gene clusters were found in *M. aquaeolei* VT8 and *A. borkumensis* [6]. Finally, because different denitrifying bacteria can use hydrocarbons, for the *Achromobacter* sp. strain LU6 and *Pseudomonas* sp. strains LU7 and LU9 phylogenetic inferences were obtained on the genes encoding NOx reductases of the denitrification pathway (Fig. 2). Interestingly, in a recent phylogenetic study the strains with high denitrification activity in saline wastewater showed a high similarity to the genera *Alcaligenes*, *Vibrio*, *Pseudomonas* and *Halomonas* [7].

This study provides insights into the diversity of cultured bacteria from a PAHs wastewater treatment area. The obtained molecular data are addressed to design new PCR-primers, probes and oligonucleotides for DNA microarray screening for the simultaneous detection of specific genes involved in the hydrocarbon metabolism. Certainly, the information obtained from culture-dependent and independent approaches complement each other in bacterial diversity studies. However, cultivation of bacteria is necessary in order to study the physiological capabilities of individual strains. Culture-based techniques are still used in microbial ecology although enrichment isolation underestimates bacterial diversity. Of special importance are catabolic genes for the hydrocarbon metabolism and we investigated them in our isolates through PCR amplification and DNA sequencing (data not shown). Further investigations on the exact role of these bacterial taxa are required. In particular, the direct utilization of a wide spectrum of organic compounds by denitrify bacteria is interesting [8] and deserves further studies. Further investigations to quantify the different denitrifying groups by fluorescence in situ hybridization (FISH) and DNA microarray are underway.

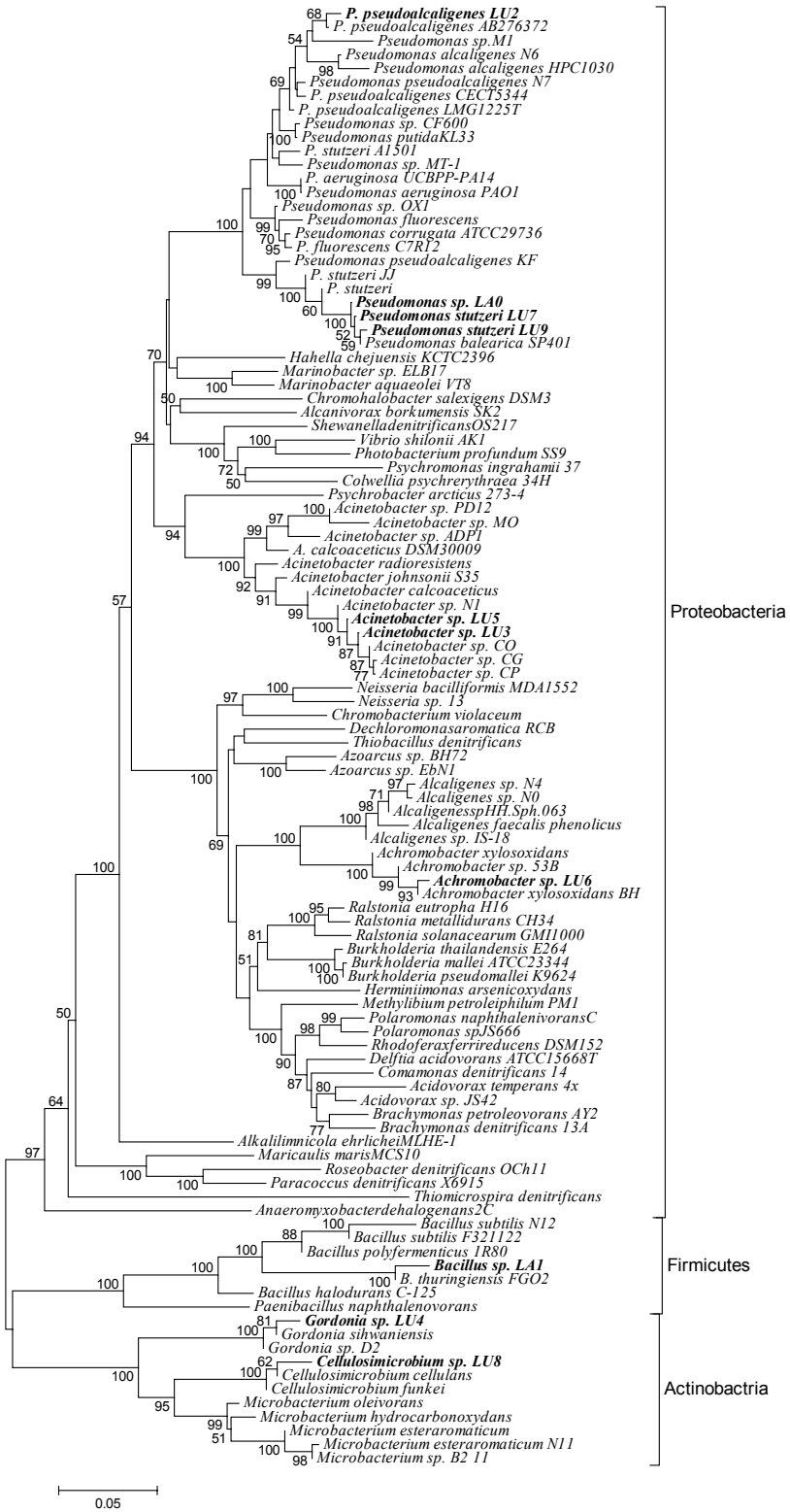

Fig. 1. Phylogenetic affiliations of the hydrocarbon-degrading bacteria from this study (highlighted in boldface). The tree was constructed by the NJ method and nucleotide substitution rates were calculated by using Kimura's two-parameter model; only values >50% are displayed.

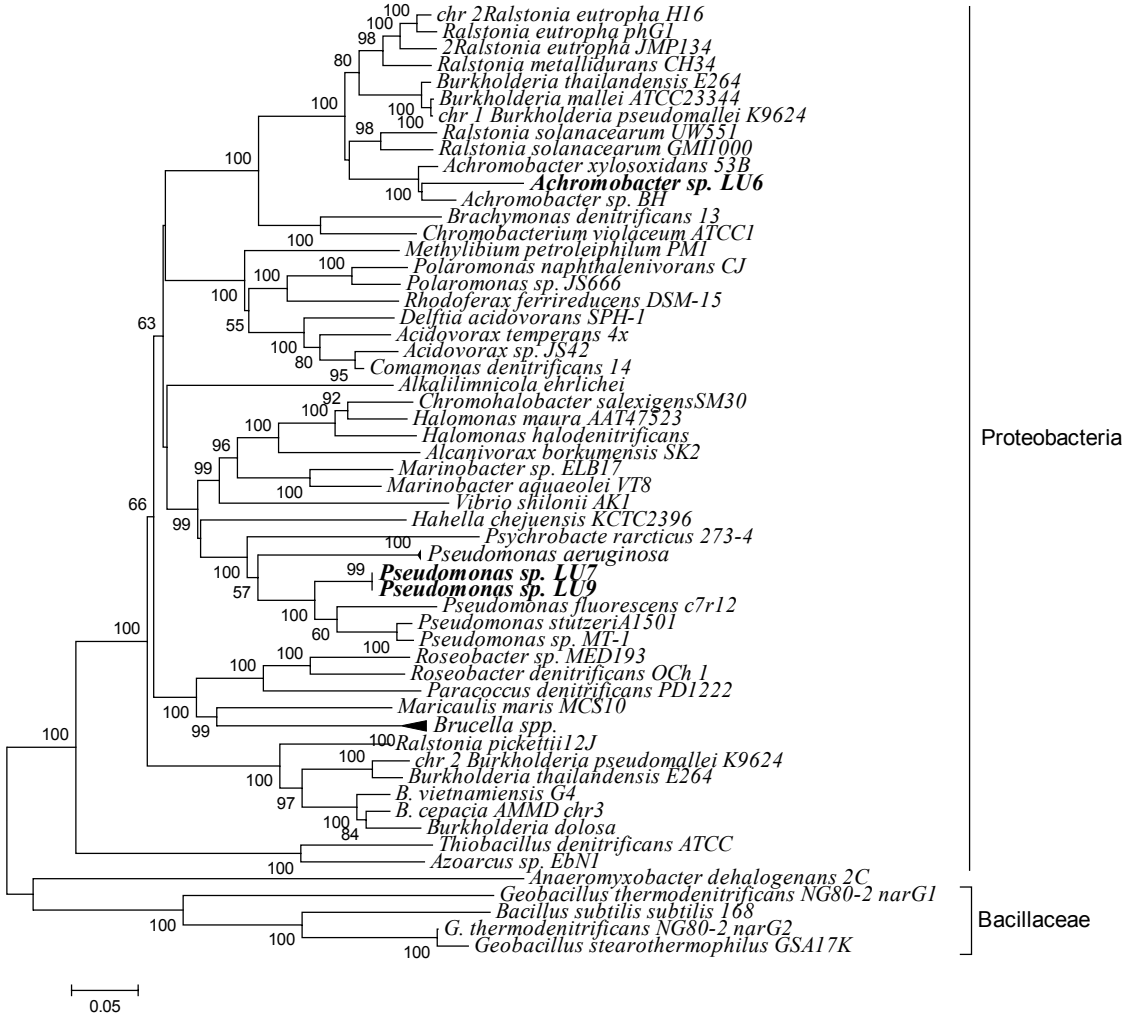

Fig. 2. Phylogenetic analysis based on the deduced residues of the nitrate reductase alpha subunit (*narG*) gene sequences from strains of the present study (in boldface) and from other α-, β-, γ- δ-*Proteobacteria* and *Bacillaceae* deposited at NCBI. The topology of the unrooted phylogenetic tree is the result of 1,000 bootstrap replications using the NJ method by pairwise deletion and Poisson correction; only values >50% are displayed. The abbreviation chr = chromosome.

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Monitoring of Bacterial Diversity in Relation to PHA Storage Capacity in an Anaerobic/Aerobic Activated Sludge SBR System

Bertan Başak[*,1], Orhan İnce[2], Nazik Artan[2], and Nevin Yağcı[2]

[1] Fatih University, Department of Environmental Engineering, 34500, Istanbul, Turkey
[2] Istanbul Technical University, Department of Environmental Engineering, 34469, Istanbul, Turkey

[*]Corresponding author: e-mail: bertan@fatih.edu.tr, Phone: +90 212 8663300 - 5552

Polyhydroxyalkanoates (PHA) are good candidates to plastics because of their complete biodegradability and material properties similar to petroleum derived plastics. They can be synthesized by numerous bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. In this study, PHA storage capacity of activated sludge were tried to increase under anaerobic/aerobic conditions and changing microbial community in the SBR has been observed by combination of molecular techniques. Polyhydroxybutyrate fraction of the inoculum sludge which was 0.23% increased to 8.1% after 5 months operation of the SBR. Corresponding values were 0.04% and 2.45% respectively for polyhydroxyvalerate. Bacteroidetes species dominated the sludge sample taken from the SBR after 5 months of operation, by 28%. Bacteroidetes followed by Proteobacteria by 17%, and Actinobacteria by 15%.

Keywords Polyhydroxyalkanoates; activated sludge; DGGE; clone library; sequence analysis

1. Introduction

Using plastics in short live applications has led to the accumulation of huge amount of non-degradable waste materials across our planet. The increased cost of solid waste disposal and accumulation of these materials in oceanic regions are some of the important environmental problems caused by plastic usage. For the past two decades, there has been a growing interest regarding the use and development of biopolymers as an ecologically useful alternative to petroleum derived plastics. Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs) have been drawing much attention, because of their material properties similar to conventional plastics and complete biodegradability. These microbial polyesters are thermoplastics with biodegradable and biocompatible properties, and the physical properties can be regulated by varying the composition of the copolymers [1]. A number of bacteria including Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, methylotrophs, pseudomonads, and recombinant Escherichia coli have been employed for the production of PHAs [2]. Even though PHAs have been recognized as good candidates for biodegradable plastics, their price compared with conventional plastics has limited their usage in a wide range of applications. Well controlled growth conditions and sterilizing substrates increase the overall cost of the PHA production by using pure cultures. Activated sludge is a good candidate for PHA production because of its cost effectiveness and simplicity. PHA production from wastewater can provide double benefits since environmentally polluting waste is converted into environmentally friendly biodegradable polymer.

PHA has an important role as carbon, energy and reducing power storage material in various microorganisms encountered in activated sludge systems and especially is known to play an important role in mixed cultures anaerobic/aerobic processing, where electron donor and availability are separated [3]. However available data are so limited about the microbial community structure of the anaerobic/aerobic process. Knowledge of involved organisms in the anaerobic/aerobic system is important for a better understanding of the mechanisms of PHA production in these systems.

In this study the Sequential Batch Reactor (SBR) was operated under anaerobic/aerobic conditions for 5 months.Carbon removal and PHA accumulating ability of the sludge have been observed. Changing microbial diversity in the reactor was also determined by molecular techniques. Relation between the PHA storage capacity and bacterial diversity of the sludge was investigated. Although PHA storage capacity of the sludge increased significantly, change in diversity was only 26%.

2. Materials and Methods

2.1 SBR operation

A SBR (working volume 8L) was utilized for culturing the activated sludge under periodic conditions. A SBR cycle consisted of 3 h anaerobic and 2 h aerobic phases of reaction, 30 min settling and 30 min withdrawing half of the volume, which was replaced with fresh medium at the beginning of the next cycle. A defined volume of biomass was removed to keep a sludge retention time (SRT) of 32 days. Timers controlled the stirring, pumps for air, medium feed and removal, and biomass removal. The reactor was operated without pH control. The temperature was kept at 20°C. The SBR was fed by synthetic wastewater of which COD concentration is 350 mg L^{-1}. Sodium acetate was the sole carbon source and the SBR feed also contained the following mineral medium: K_2HPO_4 (60 mg L^{-1}), KH_2PO_4 (30 mg L^{-1}), NH_4Cl (20 mg L^{-1}), $MgSO_4.7H_2O$ (7.5 mg L^{-1}), $FeSO_4.7H_2O$ (0.25 mg L^{-1}), $ZnSO_4.7H_2O$ (0.25 mg L^{-1}), $CaCl_2$ (1 mg L^{-1}), $MnSO_4.H_2O$ (0.2 mg L^{-1}). Influent PO_4-P and NH_4-N concentrations were 17,5 mg L^{-1}, and 5 mg L^{-1} respectively.

Samples for COD, phosphate, TSS, VSS, PHB, and PHV analysis were taken periodically at the end of anaerobic and aerobic phases to monitor system performance. PHA determination was performed according to Braunegg et al. [4] with modifications. COD, TSS, VSS, and phosphorus were measured according to Standard Methods [5].

2.2 Microbiological analysis

Genomic DNA was extracted from activated sludge samples using the FastDNA Spin Kit for Soil (Qbiogene Inc., U.K.) following the manufacturer's instructions. Amplification of 16S rDNA from the extracted DNA was performed with primers selective for the bacteria using a programmable thermal cycler. Amplified DNA was verified by by electrophoresis in a 1% agarose gel. Community profiles of bacteria within the activated sludge were obtained using DGGE analysis of PCR amplification products from primers Bact341f_GC-Bact534r as described by Muyzer et al. [6]. Electrophoresis was performed using the D-Code system (Bio-Rad) at 200 V constant current at 60°C, for 4.5 h. Gel images were recorded using a Chemi-Smart 3000 gel documentation system (Vilber Lourmat) after stained with SybrGold (1:10000) according to the supplier's instructions. Images were converted, normalized and analyzed by using the Bionumerics 5.0 software (Applied Maths). Similarities between tracks were calculated by using the Dice coefficient (SD) (unweighted data based on band presence or absence) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) and UPGMA clustering. Using Dice coefficient, a band position tolerance of 0.7% was applied for analysis. This was the minimum tolerance at which all marker lanes clustered at 100%.

For DNA clone library construction primers EUB008F and EUB1512R were used, and amplified DNA was subsequently clonned with TOPO TA cloning kit (Invitrogen). Vector inserts of the correct size were reamplified with primer pair Bact341fgc-Bact534r and were screened based on the electrophoresis position in a DGGE gel. At least one representative of every clone type in the library was sequenced Every clone type were analysed by DGGE to relate bands in DGGE profiles from the original samples with the cloned DNA. Partial 16S rRNA gene sequences were analysed and manually edited in Chromas software package version 1.45. Homology searches of the EMBL and GenBank DNA databases for the 16S rRNA gene sequences were performed with FASTA [7] provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/nucleotide.html) to identify putative close phylogenetic relatives. Sequences representing distinct phylotypes and their closest relatives were aligned. Coverage was calculated as $1 - (n_1/N)$, where n_1 is the number of clone types that was encountered only once in the library and N is the total number of clones analysed [8].

3. Results and Discussion

3.1 System performance

During this study influent COD concentration of the reactor increased gradually. System have been started to be fed by synthetic wastewater having a COD concentration of 50 mg L^{-1}, and this concentration have been increased to 350 mg L^{-1} in 88 days. Good EBPR behaviour of the reactor has been continued during almost whole operation period. Microorganism concentration in the reactor was increased depending on the increasing substrate concentration and remained stable after steady state conditions was observed. Average MLSS concentration of the reactor was about 3000 mg/L and MLVSS/MLSS ratio was approximately 0.60.

Changing concentrations of the COD and phosphorus in the reactor and accumulated PHA as mg COD L^{-1} in the microorganism during one cycle operation of SBR is depicted in Figure 1 to show typical behavior of the reactor. Anaerobic/aerobic cycle force microorganism to accumulate carbon as PHA during anaerobic phase and consume this accumulated PHA as a carbon source during aerobic phase. Microorganisms capable of accumulating enough PHA during anaerobic phase for the subsequent aerobic phase were selected during long operation period of anaerobic/aerobic conditions. Carbon source allowed entering aerobic phase cause survival of the microorganisms having no ability to accumulate PHA. Ideally, all of the influent COD is expected to be removed in the anaerobic phase. COD concentration has been dropped sharply at the beginning of the cycle and consumption rate decreased gradually. Total COD removal was 95% at the end of anaerobic phase and this ratio reached 97% at the end of the cycle. Residual COD level possibly due to microbial products generation and actually no substrate available under aerobic conditions. Phosphorus concentration in the reactor increased during anaerobic phase up to 30 mg L^{-1} and as expected started to decrease under aerobic conditions. Observed NO_3^- concentration which was less than 0.1 mg L^{-1}, showed that no pre-anoxic zone existed and conditions were completely anaerobic in the reactor for first 3 hours.

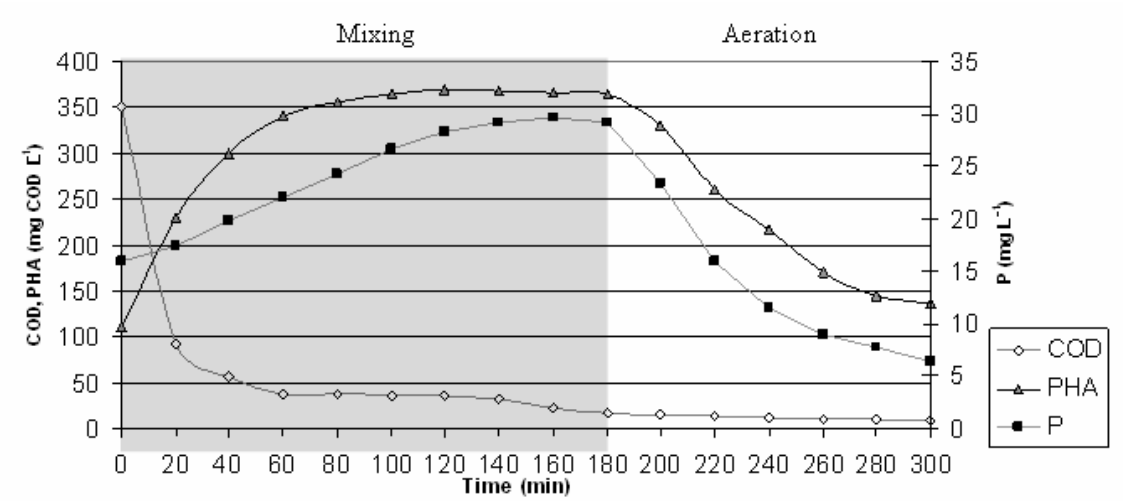


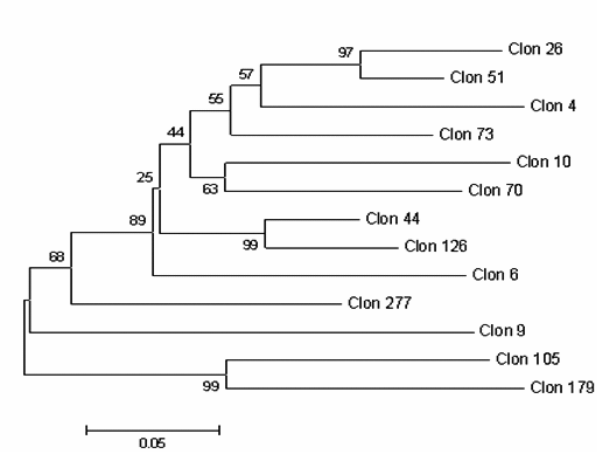
Fig 1 Typical behaviour of the SBR during one cycle as means of COD, phosphate concentrations in the reactor and accumulated PHA in the microorganisms.

Experimental results show that significant amount of the substrate entering the reactor is accumulated by microorganisms as PHA because no final electron acceptor is available under anaerobic conditions. Storing phosphorus in the form of polyphosphate under aerobic conditions and utilizing energy derived from hydrolysis of poly-P to take up carbon during anaerobic period is a survival mechanism. However possibly this was not the only mechanism, because phosphorus release was not high enough.

Microorganisms having ability to accumulate PHA were probably selected during 5 months of operation period under anaerobic/aerobic conditions. Polyhydroxybutyrate fraction of the inoculum sludge which was 0.23% increased to 8.1% after 5 months operation of the SBR. Corresponding values were 0.04% and 2.45% respectively for polyhydroxyvalerate. Polymer accumulating capacity of the biomass was observed to increase significantly.

3.2 Microbial characterization

The bacterial community was evaluated by the construction of 16S rDNA clone libraries from inoculum sludge taken at 02/09/2006 and sample taken from the SBR at 09/02/2007, and applying subsequent sequencing and phylogenetic analysis. Identified bacterial clones, their phylogenetic analysis, species similar to these clones and similarities between them were given in Figure 2 and Figure 3 for samples taken at 02/09/2006 and 09/02/2007 respectively. Approximately 16% of the sequences showed more than 97% similarity to their closest relatives on the database, similarities of the remaining were between 70% and 97%. The total number of phylotypes detected in this study was 37. The coverage of the bacterial clone libraries was 94% suggesting that the major part of the microbial diversity was recovered in this study.

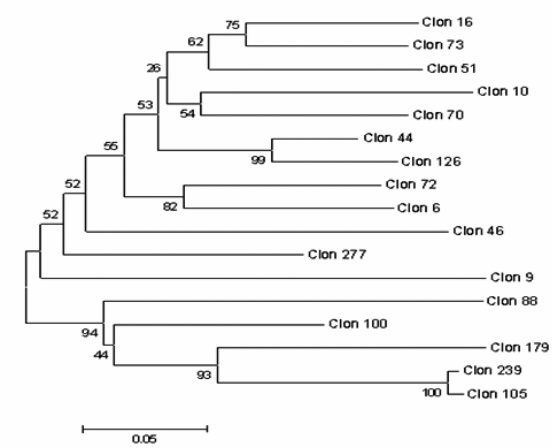


Similarity %	Species
82	Candidatus Endowatersipora
97	Frateuria aurantia
95	Herminiimonas aquatilis
90	Gamma proteobacterium SA29-B
92	Flavobacterium ferrugineum
99	Lactosphaera pasteurii
98	Hyphomicrobium W1-1B
95	Alpha proteobacterium IMCC1702
84	Geobacter sulfurreducens PCA
80	Rhodothermus marinus
99	Candidatus Microthrix parvic
82	Saprospiraceae bacterium MS-Wolf2-H
93	Flexibacter aurantiacus subsp. excathedrus

Fig 2 Phylogenetic tree of near complete 16S rDNA sequences obtained from inoculum sludge sampled at 02/09/2006

Considering all the sequences, members of the Proteobacteria dominated inoculum sludge bacterial clone library, with 12% belonging to α-proteobacteria, 5% belonging to β-proteobacteria, 5% belonging to γ-proteobacteria and 4% belonging to δ-proteobacteria. Proteobacteria followed by Bacteroidetes species by 19%, and Actinobacteria by 11%. Sequences related to Planctomycetes, Chloroflexi, and Firmicutes, accounted for 5%, 4%, and 3% respectively. The 3 bands matching with a Flavobacteria, an Anaerolineae and an α-proteobacteria showed relatively high intensity compared to other bands of DGGE image belong to inoculum sludge.

After 5 months of operation, microbial community and dominant species in the reactor were different. Similarity between communities was 74%. The 2 bands matching with an α-proteobacteria, Candidatus Endowatersipora, and a β-proteobacteria, Herminiimonas aquatilis, were disappeared. There were 7 new bands detected, 2 of them matching with 2 γ-proteobacteria, one matching with a Acidobacteria and 4 matching with 4 Bacteroidetes.



Similarity %	Species
89	Legionella-like amoebal pathogen HT99
90	Gamma proteobacterium SA29-B
97	Frateuria aurantia
92	Flavobacterium ferrugineum
99	Lactosphaera pasteurii
98	Hyphomicrobium W1-1B
95	Alpha proteobacterium IMCC1702
90	Acidimicrobidae bacterium
84	Geobacter sulfurreducens PCA
95	Bacterium Ellin6075
80	Rhodothermus marinus
89	Kaistomonas sp. IMCC1731
95	Sphingobacteriales bacterium TP498
93	Flexibacter aurantiacus subsp. excathedrus
81	Haliscomenobacter hydrossis
82	Saprospiraceae bacterium MS-Wolf2-H

Fig 3 Phylogenetic tree of near complete 16S rDNA sequences obtained from SBR at 09/02/2007

Considering all the sequences, Bacteroidetes species dominated the sludge sample taken from the SBR after 5 months of operation, by 28%. Bacteroidetes followed by Proteobacteria by 17%, and Actinobacteria by 15%. Sequences related for Acidobacteria, Planctomycetes, Chloroflexi, and Firmicutes, accounted for 1%, 4%, 3% and 2% respectively. The 3 bands matching with 3 different Bacteroidetes showed relatively high intensity compared to other bands of DGGE image. Our results from the DGGE analysis correlates with the results from the analysis of clone libraries.

4. Conclusions

The results represented here show that PHA storage capacity of the activated sludge under anaerobic/aerobic conditions increased significantly. Although microbial diversity in the reactor were changed under these

conditions, differences in PHA accumulating capability was more significant and probably most of the microorganism have adapted themselves to anaerobic/aerobic conditions.

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Potential stage in wastewater treatment for generation of bioelectricity using MFC

N.Rakesh Reddy[1], K.Nirmal Raman[1], O.K.Ajay Babu[1]and A.Muralidharan[2,*]

[1] Department of Biotechnology, SRM University, Kattankulathur, Kancheepuram dist., 603203, Tamil Nadu, India.

[2] Department of Genetic Engineering, SRM University, Kattankulathur, Kancheepuram dist., 603203, Tamil Nadu, India.

[*]Corresponding author: e-mail: srmbioenergyresearch@gmail.com, Phone: 9840096868

The Microbial Fuel Cell (MFC) technology is researched extensively and believed to be a promising source of energy in near future. Prolific research is being carried out in optimizing the efficiency with reference to design aspects while the substrate-microbial consortium deserves considerable significance. The present study was to identify a potential substrate and to characterize the microbial consortium in the wastewater sample. An economical and optimized two-chambered MFC which employs a salt bridge was used. Samples obtained from various stages of wastewater treatment plant were used as substrate. The results on comparison accounts for a maximum voltage of 1.25V and a power density of 0.02165 W/m^2 were generated from Activated Sludge sample. This substrate-microbial consortium proves to be of great potential in comparison with other samples analyzed.

Keywords Microbial Fuel Cells; Wastewater treatment; Substrate; Bioelectricity; Activated Sludge.

1. Introduction

Waste water is any water that has been affected by anthropogenic activity. Millions of gallons of waste water is produced from industries, households etc., worldwide. Hence treatment of wastewater is highly essential for maintaining a clean pollution free environment. There are a number of ways to treat the wastewater which involves a series of steps (Activated Sludge, Filtration, Clarification etc.). The power consumption in all these processes is large thereby reducing the affordability to industries. A novel method has been developed to treat wastewater as well as generate electricity. MFC has been developed in early 20th Century [10]. Microbial Fuel Cell (MFC) Technology is a potent application and serves the purpose in treating the wastewater and generating electricity.

A general Fuel cell is defined as a device which converts chemical energy in to electrical energy in presence of a catalyst. The Microbial Fuel Cell (mediator independent) utilizes the same principle but the microorganism substitutes for catalyst and its metabolism for chemical reactions.

The microorganisms oxidizes substrates (carbohydrates-glucose, dextrose etc.,) present in wastewater. During metabolism some of the energy of the substrate molecule is conserved in ATP while much remains in the products like pyruvate, acetate.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$$

Substrate is oxidized with subsequent liberation of protons and electrons. These electrons are accepted by NAD^+ molecule, which is reduced to NADH. This NADH is re-oxidized at NADH dehydrogenase, and the electrons are liberated. Subsequently the electrons follow the electron transport chain [1]. Fermentative metabolic pathways are used when no readily available electron acceptors (oxygen) are present in the bacterial environment (anaerobic). During fermentation, bacteria will deposit part of the liberated electrons on the oxidized substrate and hence form reduced metabolites such as ethanol and acetate but also hydrogen and methane reducing the efficiency of the electron transfer to the anode.

The rate of inhibition of the metabolic end products is effectual for electron transfer. As a result, the presence of oxygen in the anodic chamber inhibits transfer of electrons at the expense of water formation.

$$2H^+ + 2e^- + \tfrac{1}{2}\, O_2 \rightarrow H_2O$$

The electrons in the anodic chamber are harvested using an electrode. The flow of electrons through a conductor is facilitated by setting up a potential difference with exposure to oxygen (electron acceptor) in the cathodic chamber [14]. The protons produced in the anodic chamber are exported to cathodic chamber through a

salt bridge. The flow of electron and the potential is measured using a multimeter. Hence by this process water is obtained with production of electricity.

2. Materials and Methods

2.1 MFC Design and Operation

The microbial fuel cell used consists of dual chambers namely anodic chamber and cathodic chamber (each with volume 1litre) connected with a salt bridge. Carbon electrodes (0.5cmX12.5cm) served as anode and platinum electrodes serve as cathode. The anodic chamber was sealed with a two holed cork. The cork housed the salt-bridge and the L-tube connected to the vacuum pump facilitated anaerobic condition. The anaerobic chamber contained the wastewater sample which consisted of organic substrate. The aerobic chamber was filled with water. The two chambers were connected by means of a salt bridge to facilitate the transport of H^+ ions from the anodic chamber to the cathodic chamber. The salt bridge was made up of saturated salt solution like NaCl, KCl with agar for solidification. The circuit was closed with a resistance to measure current in a multimeter. Three identical setups with wastewater samples collected from different stages were run at room temperature. The schematic representation of the setup is given (Fig.1). Photograph of the design employed is shown below (Fig.2).

2.2 Culture and Medium

The waste water sample was collected at three different stages i.e, the effluent in anaerobic sludge digester, effluent from sand filter stage and effluent from carbon filter stage. The three effluents were run in the above mentioned experimental setups. The wastewater was collected from the wastewater treatment plant in SRM University, with the capacity of 500000 liters. The wastewater sample contains the essential nutrients supporting the growth of microorganisms and the consortia of microorganisms aiding in the process.

3. Results

3.1 Electricity Generated From Wastewater

Three separate MFC setups containing Anaerobic sludge digester, Sand Filter Effluent and Carbon Filter Effluent were run for a period of four weeks. The effective voltage and current was measured every 24 hours.

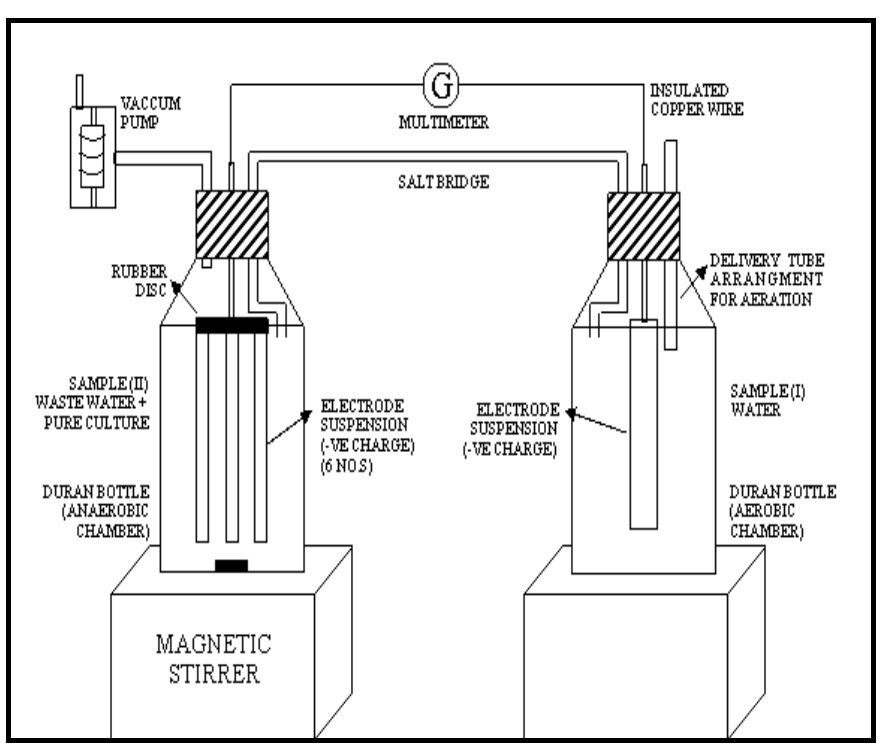

Figure 1: Schematic Representation of MFC

Figure 2: Photograph of Dual Chamber MFC

3.1.1 Generation of electricity in anaerobic sludge digester sample

The voltage obtained in this setup peaked at 1.25V after one week. A gradual increase was seen during the experiment. It reached a steady state after one week with the above stated value. The peak current produced was 0.070 mA. The power value was found to be 0.085mW and hence the power density was found to be 21.65mW/m^2. A steady voltage was obtained for the second week and a decline in voltage was observed in the third week from the inception of the experiment.

3.1.2 Generation of electricity in Sand filter sample

The voltage obtained peaked at 0.7 Volts at day 9. A linear increase was seen in this setup also. The peak current obtained was 0.027mA. The power is found to be 0.0189mW and hence the power density was found to be 4.821mW/m^2. The peak voltage attained a steady state from day 10-13 and from then on a decline in voltage was noted.

3.1.3 Generation of electricity in Carbon filter sample

A peak voltage of 0.5V was obtained at day 10. A steady increase as seen in the previous two setups was also seen in this setup. A peak current of 0.014mA was obtained. The power was calculated and found to be 0.007mW and hence the power density was found to be 1.7712mW/m^2.

A graph depicting the increase and decrease of voltage over a period of days was generated for all the three stages of sample (Fig.3).

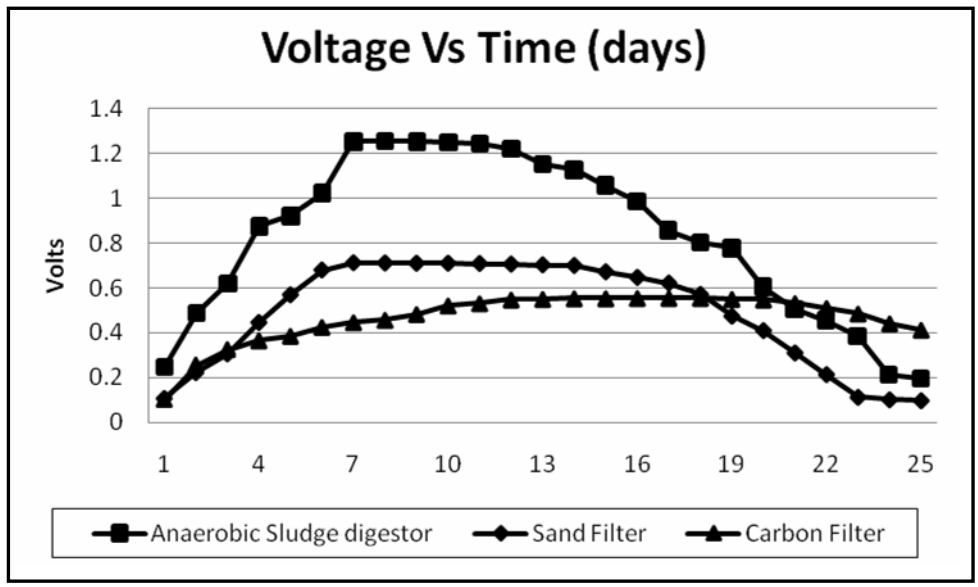

Figure 3: Voltage generated in MFC with respect to Time(days).

4. Discussion

The results obtained showed that the waste water sample obtained from the activated sludge step was found to produce better results (21.65mW/m^2) compared to the samples obtained from the samples obtained from the sand filter stage and carbon filter stage. The voltage obtained in the carbon filter sample was found to be more stable compared to the other two samples with steady peak voltage (0.5V) lasting for 8 days. The microbial population and naturally the substrate were found to be more in activated sludge compared to the other two samples. It can be inferred that the amount of substrate and population of microorganisms plays a major factor in bioelectricity production in MFC.

4.1 Consortia of microorganisms

The consortia of microorganisms found in the wastewater are large and cannot be standardized. Microorganisms such as halophiles, methanogens, and thermoacidophiles are found. The microorganism should be characterized as 'Electricigens' and 'Non Electricigens' based on its electrochemical activity. Methanogens are found to be inhibitory in nature. Microorganisms that transfers the electrons to the electrodes in the absence of mediators is much desired.

4.2 Biological oxygen Demand

The biological oxygen demand after every 5 days i.e., 5^{th}, 10^{th}, 15^{th} day is reduced by 17%, 28%, and 53% respectively. Therefore this technology also provides solution for treatment of waste water. The suspended solids found in the sample at the end of the third week were less by 60% compared to the inception of experiment.

4.3 Physical factors

The surface area of the electrode, reactor configuration, pH, ionic strength, accuracy of the measuring device, temperature, electrode performance, pretreatment of the electrodes, mixing/ agitation, dissolved oxygen content in cathodic chamber for acceptance, salt bridge resistance, external circuit resistance, etc., are the influencing factors.

Acknowledgements Authors thank the Management of SRM University for providing the team with the necessary laboratory facilities and Dr. K Ramasamy, Dean, School of Bioengineering, SRM University for his eminent guidance. The authors also express their gratitude towards Ms. Preethi Gopalan for her support rendered in executing the work.

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Production of Bioelectricity from Wastewater using stacked Microbial Fuel Cells

O K Ajay Babu[1,], Nirmal Raman K[2], N Rakesh Reddy[3], A Muralidharan[4,*]

[1-3] Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Kancheepuram dist., Tamil Nadu 603 203, India.

[4] Department of Genetic Engineering, School of Bioengineering, SRM University, Kattankulathur, Kancheepuram dist., Tamil Nadu 603 203, India.

[*]Corresponding author: e-mail: srmbioenergyresearch@gmail.com, Phone: 9840096868

Microbial fuel cell (MFC) is a device which converts chemical energy in the organic substrate into electrical energy using microorganisms as a catalyst. The preliminary studies were conducted in optimizing the design of the MFC to maximize the production. Such MFC models were stacked to enhance the bioelectricity production. A comparative study was carried out to test the effectiveness in series and parallel. A maximum voltage of 3.2 volts (connected in series) and 1.1 volt (connected in parallel) was obtained. Maximum power of 0.288mW was obtained in stacked MFC with series connection. MFC performed dual role of bioelectricity production and treatment of wastewater. MFC has a potent application of reducing the cost of operating wastewater treatment plants.

Keywords Microbial fuel cells (MFC); Renewable Energy; Mediatorless; Wastewater treatement; Bioelectricity

1. Introduction

The renewable forms of energy are more prominently pronounced due to the depletion in the non-renewable forms of energy. Research in this field is very popular owing to this fact. Major efforts are devoted to develop alternative methods of electricity production. Electricity production from renewable resources without a net carbon dioxide emission is much desired. Microbial Fuel Cells (MFC's) represent a new form of renewable energy by converting organic matter into electricity using bacteria as a catalyst [9]. Millions of gallons of waste water is produced from industries, households etc., worldwide. Using wastewater which has high organic content would be ideal as a substrate in MFC. Treatment of wastewater is highly essential to maintain a clean pollution free environment. Therefore, this method serves to be novel in generating electricity and also treating wastewater.

The process of microbial metabolism is complex, involving many enzyme-catalyzed reactions. It progresses through a series of intermediates involving successive redox reactions, and in this respect resembles an electrochemical process. In normal microbial catabolism, a substrate such as carbohydrate is oxidized initially without participation of oxygen, and electrons are released by enzymatic reactions.

These electrons are stored as intermediates which are used in Redox reactions: as a result, energy is released and is used to fuel further reactions for the living cell for maintenance and growth via bio-synthetic reactions. The ultimate "electron-sink" is molecular oxygen [1].

The large harvest of electrons is stored as reduced intermediates, but the eventual terminus in the respiratory chain is oxygen:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \; [E_o = 0.014V] \qquad (1)$$

$$6O_2 + 24H^+ + 24e^- \rightarrow 12H_2O \; [E_o = 1.23\ V] \qquad (2)$$

In a microbial fuel-cell, anaerobic metabolism must be promoted at the anode in order to convert organic matter to electricity in an effective manner. Fermentation is the well known mechanism for anaerobic digestion, but fermentation resultant products do not readily react with the electrodes, thereby retaining the electrons. Effective anaerobic oxidation of complex assemblages of organic matter, such as those found in most wastes and biomass, requires the fermentation products from the metabolism. In addition, other constituents, such as aromatic compounds and long-chain fatty acids, are to be oxidized with electron transfer to an acceptor. In microbial fuel cells the fuel source is generally microbial degradable organic matter. Microorganisms catalyze and release electrons from organic matter and transfer them to various electron carriers that are electrochemically active. Separating the microorganisms from the source of oxygen in a MFC intercepts the

flow of electrons to oxygen which microorganisms would catalyze if oxygen were available. This facilitates the transfer of electrons to the anode.

There are two ways in which microorganisms can transfer electrons to the anode electrode: using mediators like Thionone, Neutral Red etc. and mediators produced by Bacteria itself (direct transfer of electrons through respiratory system through membrane bound Cytochrome). *Shewanella putrefaciens, Geobacter sulfurreducens, Geobacter metallireducens* and *Rhodoferax ferrireducens* are found to directly transfer electrons to the anode (do not require mediator based systems) [10].

2. Design of Microbial Fuel cell

2.1 Electrodes and Electrode chambers

Microbial Fuel Cell is a dual chambered system namely Anodic chamber and Cathodic chamber. Both the anode and the cathode are made of carbon electrodes of size 12.5cm X 0.5cm (Cylindrical). The effective surface area was increased by placing two electrodes in both the chambers. The volume of each chamber with the electrodes was one liter. The top of the anodic chamber was sealed with three holed rubber stopper which houses the salt bridge, the electrode, and an inlet pipe. The entire set up was sealed again with wax and sparged with CO_2 to ensure strict anaerobic set up and further connected to vacuum pump to remove gas from headspace. The cathodic chamber (aerobic chamber) sparged with oxygen served as an electron acceptor. The design is shown as a schematic representation (Fig.1). Photograph of the design employed is shown below (Fig.2).

2.2 Medium and Inoculum

The experiments were carried with three different substrate fuel sources. **Synthetic Wastewater medium** (glucose, 3 g/l; NH_4Cl, 0.5 g/l; H_2PO_4, 0.20 g/l; K_2HPO_4, 0.20 g/l; $MgCl_2$, 0.25 g/l; $CoCl_2$, 20 mg/l; $ZnCl_2$, 10 mg/l; $CuCl_2$, 10 mg/l; $CaCl_2$, 4 mg/l; $MnCl_2$, 10 mg/l) [16]. The bacterial inoculum obtained by inoculating 5% (w/v) from dewatered sludge (85% water) was used for the synthetic waste water medium. Wastewater from **Lamella Separator**, **Anaerobic Sludge Digester** from SRM University Wastewater Treatment Plant was collected. The microbes (natural consortium) in the wastewater and sludge served as inoculum.

2.3 Experimental Conditions and External Circuit

The experiment was conducted at 30^{o}C. The system employed a salts bridge which connected the anodic and cathodic chambers. The salt bridge was prepared with saturated salt solution (NaCl, 5% agar for solidification). Electrons flow from the anode to the cathode through an external circuit. The net resistance (external resistance + internal resistance) was found to be varying between 17KΩ to 25KΩ.

3. Biological Mechanism

Microorganisms are made completely devoid of oxygen in the anodic chamber, and hence the flow of electrons to oxygen is intercepted. Under these circumstances the electrode acts as the electron acceptor and the electrons are transported through the external circuit, forming water in cathodic chamber. The anode and cathode chambers are separated by a salt bridge that restricts oxygen diffusion from the cathodic to the anodic chamber while allowing the protons released from metabolism of organic matter to move from anode to cathode. At the cathode, electrons, protons and oxygen combine to form water.

The oxidation/reduction process in anodic chamber produces a hydrogen gradient and allows hydrogen-protons to diffuse to the cathode portion through the salt bridge. The cathode portion also consists of this oxidation/reduction process; however, since the cathode allows oxygen to diffuse from the sparged air, water is formed. The energy available from the proton gradient due to the anode is harnessed by connecting an external circuit from the anode to the cathode to allow the flow of electrons.

4. MFC Tests

MFC with synthetic wastewater as the substrate was inoculated with 5% inoculum (de-watered sludge) and other waste substrates were run with the above-mentioned similar setups. The individual potentials were measured to test the efficiency. The above setups were duplicated in triplets and were connected in series and in parallel. The resultant potential was calculated as single output and the effectiveness was tested. The readings

were recorded after three days considering the period for acclimatization and growth of the Bacterial Consortia when synthetic medium was used. The voltage was recorded at regular intervals of 24hrs.

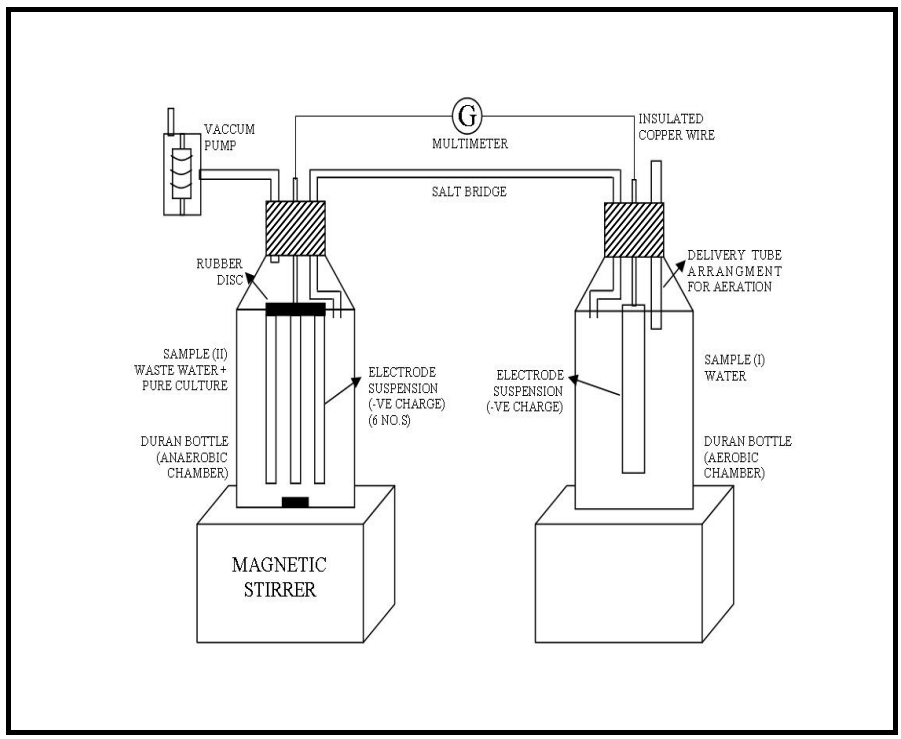

Figure 1: Schematic representation of a dual chambered MFC with salt bridge.

Figure 2: Photograph of Dual Chamber MFC

5. Calculation and Analysis

The voltage (V) produced in the setup was measured using a multimeter. It was then used to calculate the power using the formula, **P=VI**. The current (I) generated was calculated using Ohm's Law, **V=IR.** Power Density (PD) calculated using the formula, **PD=P/A,** where A is the surface area of the anode (m^2).

6. Results

The peak and steady results were obtained for a period of 8 days and the values are tabulated (Table 1).

Table 1: Comparison of results from various substrates used

Substrate	Voltage (V)	Current (mA)	Power (mW)	Power Density (mW/m^2)
Synthetic Wastewater	1.563	0.070	0.1094	27.35
Wastewater from Lamella Separator	0.7	0.027	0.0189	4.725
From Anaerobic Sludge digester	1.25	0.070	0.0875	21.87

6.1 Generation of electricity in MFC stack

Efficient results obtained in Synthetic medium were attributed to availability of substrate and uniform consortia. The synthetic medium was scaled up in triplicates in 2 sets which were circuited in parallel and series.

6.2 Generation of electricity in MFC stack

6.2.1 In series and In parallel

A maximum voltage of 3.2 Volts and 1.1 Volts and a maximum current of 0.09mA and 0.25mA were obtained in series and parallel connections respectively. It produced a power of 0.288mW in series and 0.275mW in parallel. Each MFC produced a maximum voltage of 1.2 Volts and the current obtained was 0.1mA. In series, the effective voltage obtained was found to be sum of all the individual voltage obtained in each cell with minor losses and the current obtained was found to be average of current obtained in the three cells. In Parallel, the effective current was found to be the sum of three cells and effective voltage was the average of the values obtained in three cells. Graph representing the increase and decrease of voltage over a period of days were generated (Fig.3).

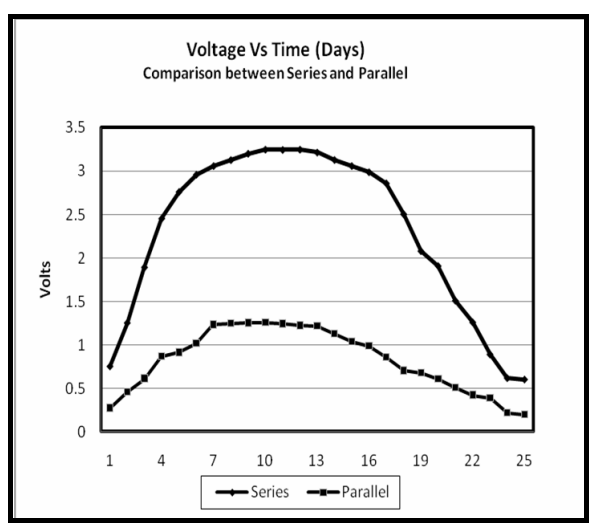

Figure 3: Voltage Vs Time in Series and parallel connections.

7. Discussion

7.1 Consortia of microorganisms

The consortia of microorganisms found in the wastewater are large and cannot be standardized. Microorganisms such as halophiles, methanogens and thermoacidophiles were found. Microorganisms play a major factor in MFC due to the reason that, only few species microorganisms have electrochemical properties (electricigens). When anaerobic sludge is used as an inoculum for MFCs, enrichment strategies should be devised to limit the growth of methanogens and to enhance the growth of the electrochemically active bacteria on the anode. The microorganisms with electrochemical property have the ability to transfer the electrons to the anode surface. Hence undesired microorganisms which lack this property may inhibit electron transfer thereby reducing electricity generation. Hence, novel methods have to be developed to select the microorganisms with the above-specified properties. Almost in all set ups at least 76h lag has been reported. This can be minimized by acclimatizing the culture with the substrate and research also suggests that growing a bio-film on the anode can increase the power density.

7.2 Surface area and position of the Electrodes

The surface area of electrodes plays a major role in generation of electricity in MFC. The increase in the surface area enhances the electron transfer. Increasing the surface area by adding an electrode to the anodic chamber does not result in doubling of voltage. The position of the electrode in the cathodic chamber also determines the redox potential in formation of water.

7.3 Reactor Configuration

In the current study a batch reactor (1litre Reagent Bottle) was used. The batch reactor is an ideal selection for maintaining anaerobic conditions for a long period of time. The decrease in voltage was attributed to the decrease of substrate which in turn resulted in decline of microbial population. To negate this problem, additional substrate i.e. wastewater has to be added and subsequent removal of the residual wastewater has to be facilitated to maintain the maximum voltage obtained. But this method would compromise the anaerobic conditions in the reactor. Hence it can be inferred that an anaerobic fed-batch reactor would be an ideal choice for MFC. The reactor volume was also significant in production of bioelectricity. Trails carried out in lesser volume reactors (250ml, 500ml) generated lower voltage compared to 1 liter reactor used in the current study. It can be deduced from the experimental results that scale up of the reactor could produce better results. Constant stirring/mixing also influences the voltage that is generated.

7.4 Ionic Strength, pH, Temperature

In an experiment conducted with different concentration of NaCl (50mM, 100mM, 150mM….300mM), there was a considerable level of increase in the power density (up to 60%). It can be concluded that the power density is also a function of ionic strength. With increase in ionic strength, the power density increased. The conductivity of the solution can be increased within the permissible level which is suitable for the growth of the bacteria. However, the effect of temperature and pH is not that prominent but metabolic enzyme sensitivity is critical and should be considered.

8. Conclusion

High voltage is obtained in synthetic wastewater sample in comparison with the other substrates used. But, the sample (substrate) used from anaerobic sludge digester resulted 80% of the potential generated from the synthetic sample. Therefore

1. The sample from anaerobic sludge digester is ideal stage in waste water treatment to serve as substrate for MFC.
2. The MFCs can be stacked in series to increase the output.
3. There are several factors that could be responsible for low electron and energy recoveries in MFCs. The factors are directly or indirectly dependent on each other. A complete optimization of each and every factor is critical.

The large scale implementation of MFC will require novel strategies to be developed. Further improvements can be made to MFC configurations to improve energy recovery or to increase voltages by linking MFCs in series. This can result in new technologies that will make electricity generation using MFCs a practical method in wastewater treatment.

Acknowledgements Authors thank the Management of SRM University for providing the team with the necessary laboratory facilities and Dr. K Ramasamy, Dean, School of Bioengineering, SRM University for his eminent support. The authors also express their gratitude towards Ms. Preethi Gopalan for her help rendered in executing the work.

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Seasonal dynamics of bacterial population degrading dimethylarsenic acid in Lake Kahokugata

Wakana Hirota[1], Teruya Maki[1], Tastuya Kakimoto[1], Takashi Shimada[1], Hiroshi Hasegawa[1], and Kazumasa Ueda[1]

[1] Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa, 920-1192, Japan.

The degradation processes of organoarsenic compounds in aquatic environments would depend on the bioactivities of microorganisms and significantly influence the cycles of the arsenic species. The concentrations of arsenic species were determined in Lake Kahokugata. During the all season, the inorganic arsenic was detected, while the dimethylarsinic acid (DMAA) appeared in only winter seasons, and MMAA was not detected. Moreover, when DMAA was added to the water samples of Lake Kahokugata collected at every month from June in 2005 to November in 2006, the DMAA in the sample water of all seasons decreased and was converted to inorganic arsenic until the 28th day of incubation. According to the Most Probable Number (MPN) procedure, the DMAA-degrading bacteria were detected at cell densities ranged from 120 cells/mL to 2100 cells/mL during the all season. These facts suggested that the lake water of all season in Lake Kahokugata possessed the microbial activities for DMAA decomposition.

To determine the composition of DMAA-degrading bacteria, the total 352 isolates obtained as dominated bacterial species were analyzed by the restriction-fragment-length polymorphism (RFLP) analysis of 16S rDNA genes. As a result, total 352 isolates were classified into 10 types of which the rates of total isolate numbers indicated seasonal changes. The diverse compositions of DMAA-degrading bacteria would seasonally change and control the organoarsenic degradation contributing to the seasonal arsenic cycles in Kahokugata.

Keywords organoarsenic, dimethylarsinic acid, DMAA-degradation, bacterial population, MPN, RFLP

1. Introduction

In aquatic environments, arsenic compounds are widely distributed in a variety of chemical forms, and some of them are known to endanger human health and organism activities at high concentration. The dynamics of arsenic species depend primarily on the bioactivities of microorganisms that readily metabolize the arsenic species[1, 2].

Arsenate is a chemical analogue of phosphate and may interfere with oxidative phosphorylation[1]. Accordingly, microorganisms, such as phytoplankton (microalgae) and bacteria, uptake and accumulate ambient arsenate under phosphate-limited conditions through their phosphate-metabolic mechanisms[3]. Moreover, the microorganism cells reduce to arsenite and methylate it into monomethylarsonic acid ($CH_3AsO(OH)_2$; MMAA(V)) and dimethylarsinic acid ($(CH_3)_2AsO(OH)$; DMAA(V))[4]. The produced MMAA and DMAA are subsequently converted to more complex organoarsenic compounds, such as arsenobetaine or arsenosugars[4].

The degradation process of organoarsenic compounds also can not be neglected in considering field data of arsenic cycle in aquatic environments[5]. The seasonal change of the DMAA-degrading bacterial communities in the Lake Kahokugata[6] and Lake Kibagata[7] have been investigated. These findings indicate that these are many species of bacteria that have a degradation activity of the organoarsenic compounds that are ubiquitous in aquatic environments. However, these studies were undertaken on the dynamics of bacterial composition during only 6 months and the bacterial composition of winter season is uncovered.

In this study, we investigated the DMAA degradation process in lake water samples using chemical analytical method, and the dynamics of microbial degradation activities. Moreover, the DMAA-degrading bacteria and the species composition during the investigation period were also assessed using the most-probable-number (MPN) procedure and the restriction-fragment-length polymorphism (RFLP) analysis of 16S rDNA sequences[8]. DMAA was selected as a representative of organoarsenic compound that is widely distributed in freshwater[9].

2. Materials and Methods

2.1 Sampling

Surface lake water sample was collected in polycarbonate bottles (2000 mL) from Lake Kahokugata in Ishikawa Prefecture of Japan, from May 2005 to January 2007. One thousand Five hundred milliliter of sample water

provided for measurement of DMAA degradation activities of natural lake water. Moreover fifty milliliter of sample water was directly used for enumerating DMAA-degrading bacteria with the MPN procedure.

2.1 Experimental design and biodegradation of DMAA in lake water

Several polycarbonate bottles (500mL) were filled up with the lake water, and were transferred to our laboratory. Within 2 hours after the sampling, DMAA (nacalai tesque, Kyoto, Japan) solution were added into three bottles at a final concentration of 1 μmol/L and the bottles were incubated at 20 ºC under dark. The air phases in the bottles kept to be as minimum as possible, because a previous experiment resulted that biodegradation and mineralization of DMAA preferred dark and microaerobic-anaerobic conditions to light and aerobic conditions[10]. During the 56 days of incubation, portions (10 mL) of water samples were collected every 7 days, and used for determining the arsenic compounds concentration. To determine the arsenic compounds, the cold-trap HG-AA speciation procedure was employed.

2.2 MPN counting for DMAA-degrading bacteria

Cell numbers of DMAA-degrading bacteria were estimated by an MPN procedure with a modified CD (Czapec-Dox) medium, which contained 1 μmol/L of DMAA as a carbon source instead of sucrose[8]. The modified CD medium was composed of $NaNO_3$ (2 g/L), K_2HPO_4 (1 g/L), $MgSO_4$ $7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$, thiamine-HCl (0.05 mg/L), and DMAA (0.214 mg/L) in the filtered and autoclaved lake water of Kahokugata. The bacteria grown in this medium are considered to metabolize DMAA and are defined as DMAA-degrading bacteria. The lake water samples were serially diluted in 10-fold with the autoclaved lake water down to 10^{-5}, and then 10 mL of the modified CD medium was added to each of the diluted aliquots (10 mL). After being thoroughly mixed, each diluted aliquot was dispensed into 24 wells on a 96-well microtiter plate (Microtest^{TM}96: FALCON, NJ, USA) at the volume of 200 μL each. The microtiter plate with tightly sealed were incubated at 20 °C under dark condition. After the 14 days of cultivation, bacterial growth in each well was assessed by the turbidity (OD660 > 0.05). Most probable numbers of DMAA-degrading bacteria were estimated from the numbers of bacterial growth positive wells in each dilution series using a macro program in Microsoft Excel 11.2[11].

2.3 Classification and identification of DMAA-degrading bacteria using 16S rRNA gene information

The DMAA-degrading bacteria were isolated from the positive wells at as high dilution series as possible, and were classified roughly by RFLP analysis of their 16S rDNA genes[12, 13]. After the bacterial isolates were incubated, the bacterial cells were collected using the centrifugation. The genomic DNAs of bacterial cells were extracted using SDS, proteinase K, and lysozyme as described previously[11]. Furthermore, the genomic DNAs were purified by phenol-chloroform extraction, chloroform extraction, and ethanol precipitation[11]. The 16S rDNA fragments (ca. 1450 bp) of isolates were amplified by polymerase chain reaction (PCR) using oligonucleotide primers, 27F and 1492R[14]. Thermal cycling was performed using a Program Temp Control System PC-700 (ASTEC, Fukuoka, Japan). The resulting PCR products were digested with an endonuclease mixture, *Pst* I and *Hinf* I (TOYOBO CO., LTD., Osaka, Japan), and separated in 1.5% w/v agarose gel-LE of the classic type (Nacalai Tesque, Kyoto, Japan).

3. Results and Discussion

3.1 Seasonal variation in Lake Kahokugata

The abundance of DMAA degrading-bacteria estimated by the MPN method with the modified CD medium which included 1 μmol/L DMAA as a sole carbon source fluctuated between 120 cells/mL and 910 cells/mL during the investigation period (Fig. 1). The concentrations of Inorganic arsenic of Lake Kahokugata ranged from 2.8 nmol/L to 23 nmol/L and DMAA was detected from winter season to spring season at the concentrations ranging from 1.4 nmol/L to 13 nmol/L. MMAA were not detected. The low amount of DMAA indicated the demethylation of DMAA by microorganisms in aquatic environments. The biodegradation of DMAA and MMAA by bacteria may control arsenic cycling in aquatic ecosystems.

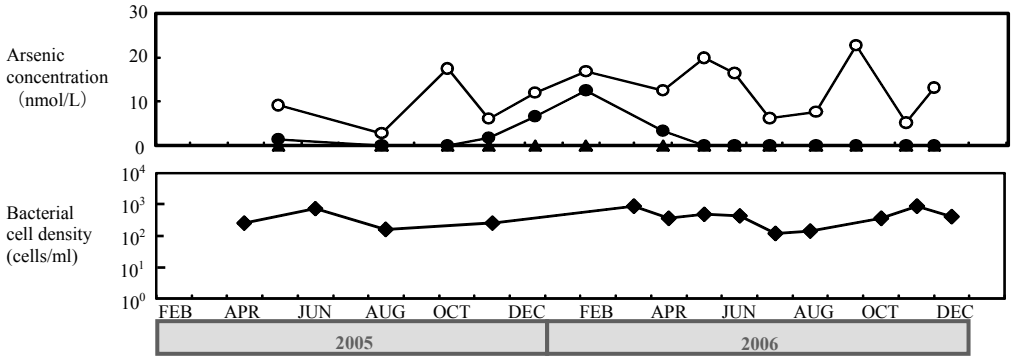

Fig. 1 Seasonal variation in the cell density of DMAA degrading bacteria and the concentrations of arsenic species. (a) Closed diamond indicate the cell density of DMAA-degrading bacteria estimated by MPN procedure. (b) Open circles, closed circles and closed triangles indicate the abundance of inorganic arsenic, DMAA and MMAA, respectively.

3.2 Biodegradation of DMAA in the lake water

The biodegradation process of DMAA was investigated after the lake water sample from Kahokugata was artificially contaminated by DMAA at the final concentration of 1 μmol/L. the concentration of DMAA onset of the experiment decreased from over 900 nmol/L until the first 28 days of incubation, and fluctuated below a concentration of 290 nmol/L until 42 days of incubation (Fig 2). In accordance with the decrease of DMAA, the concentration of inorganic arsenic compounds, which are considered to be the resultant products from DMAA biodegradation, increased from below 10 nmol/L to 520 nmol/L at least until the first 35 days, and fluctuated over the concentration of 650 nmol/L until 42 days of incubation. MMAA was below the detection limit. There is no seasonal pattern of DMAA degradation in the lake water. The DMAA- and MMAA-degrading bacteria generally inhabiting in aquatic environments, for example in Lake Kahokugata, might degrade the methylarsenic compounds produced by microalgae and contribute to the arsenic cycling in aquatic ecosystems.

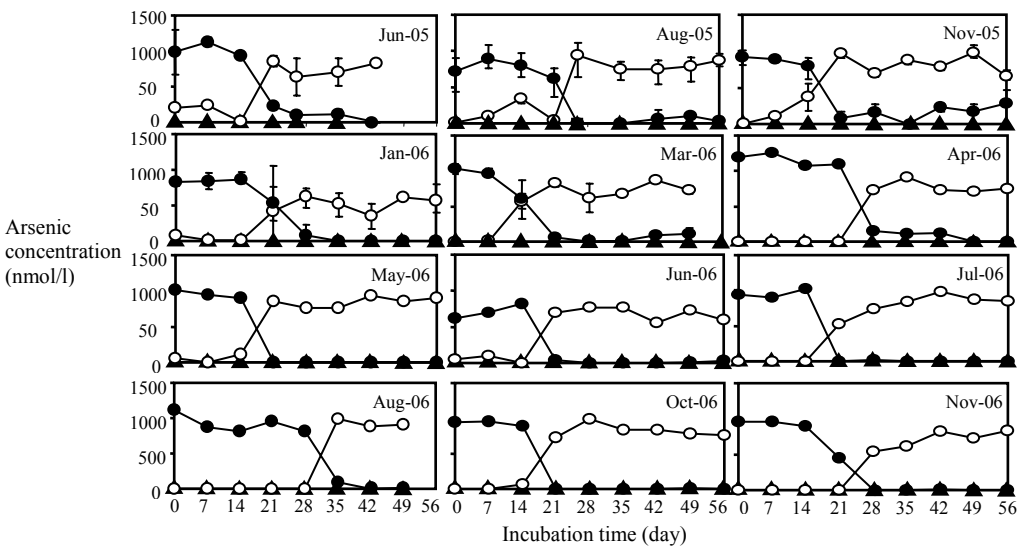

Fig. 2 Changes in concentrations of arsenic compounds in lake water samples, to which 1 μmol/l of DMAA have added. The lake water samples were collected from Lake Kahokugata at every few months from June in 2005 to November in 2006.

3.3 Classification and identification of DMAA-degrading bacteria using 16S rDNA information

Total of 352 strains of DMAA-degrading bacteria were isolated from the MPN cultures at the highest dilution series. After PCR-amplification of their 16S rDNAs, the DMAA-degrading bacterial isolates were classified into 10 ribotypes by the restriction fragment length polymorphism (RFLP) analysis. The appearances of the RFLP type groups seasonally fluctuated in Lake Kahokugata (Fig. 3).

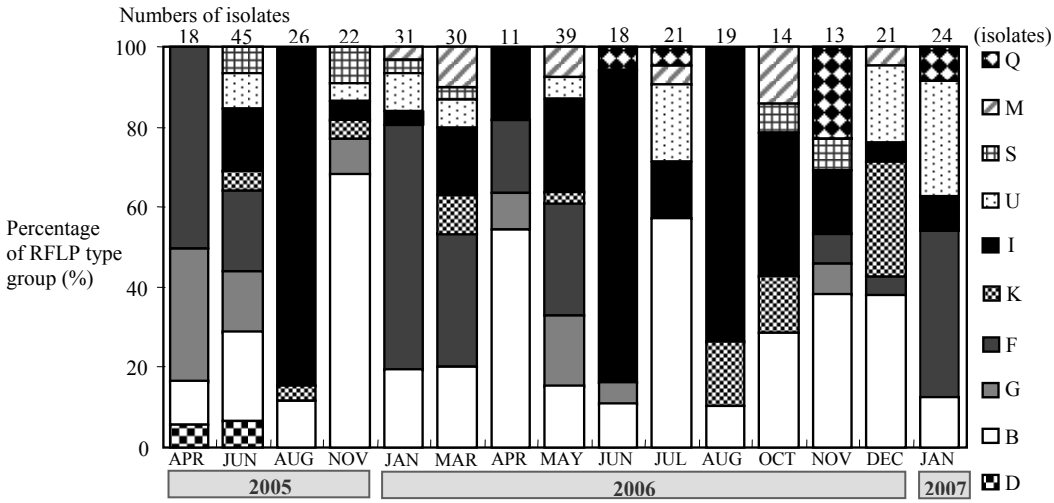

Fig. 3 Seasonal change in percentage of each RFLP type groups from April in 2005 to January in 2007.

DMAA-degrading bacteria belonging to Type B constantly occupied the percentages ranging from 11 % to 68 % of total isolates during the investigation period. The isolates of Type G were detected from spring season to summer season at the percentage of RFLP type group ranging from 5.6 % to 33 % of total isolates. The isolates of Type I significantly dominated in summer season of every year indicating the percentage of RFLP type group up to 85 % of total isolates. The isolates of Type F appeared from fall season to winter season occupying from 4.8 % to 61 % of total isolates, and disappeared in summer season of every year. It shows the bacterial population contributing to DMAA-degradation sequentially changes among the seasons. Methylarsenic degradation percentages depending on bacterial activities were reported to show seasonal changes of methylarsenic species in natural waters[15]. The dominant bacterial population which detected in this study possibly contributed to the seasonal cycle between inorganic arsenic and methylarsenic species. However, the degrees of the DMAA-degradation activities in the lake water maintained a similar level during investigation period, suggesting that the bacterial composition would not influence the degradation degrees in the lake water.

4. Conclusion

The DMAA-degrading bacteria would contribute to the arsenic cycling in Lake Kahokugata. DMAA which was added to the lake water was degraded, and was converted into inorganic arsenic within 35 days of incubation under dark condition. Moreover, the isolates of DMAA-degrading bacteria were obtained from the lake water at the call densities ranging from 120 cells/mL to 910 cells/mL. Accordingly the DMAA-degrading bacteria would contribute to the arsenic cycling in Lake Kahokugata. Based on the 16S rDNA-RFLP analysis, the DMAA-dagradation bacterial population was composed of 10 RFLP types. Some type was constantly detected, while some types were seasonally detected. It indicated that the composition of DMAA-degrading bacteria changes seasonally.

Acknowledgements This research was supported by a Grant-in-Aid for Encouragement of Young Scientists (17710061) from the Ministry of Education, Science, Sports and Culture. The Nissan Science Foundation supports this work. This work was supported by the Global Environment Research Fund (RF-072) provided from the Ministry of the Environment, Japan.

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Sewage bacteriophage photoinactivation by porphyrins immobilized in solid matrixes

L. Costa[1], C. M. B. Carvalho[1,2], J. P. C. Tomé[2], M. A. F. Faustino[2], M. G. P. M. S. Neves[2], A. C. Tomé[2], J. A. S. Cavaleiro[2], Z. Lin[3], J. P. Rainho[3], J. Rocha[3], Â. Cunha[1], and A. Almeida[1,*]

[1] CESAM and Department of Biology, University of de Aveiro, 3810-193 Aveiro, Portugal
[2] Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[3] CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

[*]Corresponding author: aalmeid@ua.pt, Phone: +234 370784

Photodynamic therapy is a platform technology which uses a combination of a photosensitizer, light and molecular oxygen to achieve selective destruction of a biological target. This methodology is already in use for the inactivation of microorganisms but its application in wastewater disinfection is incipient. In this work we tested the effect of three solid matrixes with immobilized porphyrins in the photoinactivation of a sewage bacteriophage. The phage inactivation to the limits of detection (reductions of about 7 log) with one of the tested materials, means that this insoluble material can be applied in wastewater disinfection with the same efficacy of the non-immobilized photosensitizer. The complete eradication of viruses with low light intensity means that this technology can be applied to wastewater disinfection under natural irradiation conditions during all year, including the cloudy days of winter. In addition, this is an inexpensive and easily applicable methodology.

Keywords photodynamic therapy; porphyrins; bacteriophages; wastewater; immobilized photosensitizers; solid matrixes

1. Introduction

The lack of safe and efficient technologies for wastewater treatment is one of the causes responsible for the reduction of water resources and the increase of environmental pollution. From the human perspective, water has an important implication in the transmission of infectious diseases, being necessary to develop new technologies to get polluted waters reusable and ensure the preservation of waters.

Porphyrin compounds associated to photodynamic antimicrobial chemotherapy (PACT) can be a promising chemical disinfectant for the eradication of pathogens as they are effective in inactivating microbial cells [1]. When photosensitizers are exposed to light in the presence of oxygen, they produce singlet oxygen and free radicals which are cytotoxic to pathogen population with very limited damage to the host tissue [2,3].

Soluble photosensitizers are reported as efficient compounds to carry out PACT experiments [4-9]. This is far from appropriate for applications to water disinfection, where residual traces of photosensitizer in the water output would certainly not be acceptable. On the other hand, the use of non-recoverable photosensitizers might not only introduce residual traces of sensitizer, but also turn this technology a high-cost one. The complete removal of the photosensitizer from the treated water is thus a very important issue. Since removal of soluble photosensitizers from water is extremely difficult, this problem can be solved by using photosensitizers bound to insoluble solid matrixes. This raises a new problem which is to find photosensitizers that after being immobilized are still active. In this work we show that immobilised porphyrins can be very active in the photoinactivation of somatic sewage bacteriophage and thus can be interesting materials to inactivate pathogenic microorganisms present in water or wastewater.

The aim of this study was to investigate the effect of porphyrinic photosensitizers coupled to insoluble materials on the photoinactivation of a somatic sewage bacteriophage of *Escherichia coli* (T4-like phage).

2. Material and Methods

Photosensitizers

In this work, three hybrid materials (Materials 1-3) for bacteriophage inactivation were prepared by immobilization of porphyrin derivatives on two different solid matrixes. The effect of these photosensitizing materials, at different concentrations of photosensitizer (5, 20 and 100 μM), was evaluated in laboratory conditions under white light irradiation (40 W m^{-2}). The photosensitizer not immobilized was used at 0.5, 1.0 and 5.0 μM for comparison.

Experimental set up

The efficiency of the three insoluble materials was evaluated through quantification of the number of bacteriophage in laboratory conditions. The suspension of phages was diluted on phosphate buffer (PBS) until 5 x 10^7 PFU mL^{-1} (1000 times higher than that of residual waters) and distributed in 600 mL acid-washed and sterilised glass goblets (20 mL per each of 8 goblets). Adequate amounts of the hybrid material were added to three goblets in order to obtain 5, 20 and 100 μM of photosensitizer. In another goblet the non-immobilized photosensitizer (5 μM) was added for comparison with the immobilized one. The other four goblets were used as dark, light and support material controls (in light and dark conditions). In the light control no photosensitizer material was added but the goblet was exposed to the same irradiation protocol. In the dark control, the hybrid material was added (until 100 μM of photosensitizer) and it was covered with aluminium foil. In the material controls, the support material without the photosensitizing agent was added and one was exposed to the same irradiation protocol (light support material control) and the other was covered with aluminium foil (dark support material control). The test goblets and light controls were exposed in parallel to 40 W m^{-2} white light (PAR radiation, 13 lamps OSRAM 21 of 18 W each one, 380-700 nm), at 20-25ºC, during 270 minutes, under stirring (100 rpm). Sub-samples of 1 mL were taken at time 0, 30, 60, 90, 180 and 270 minutes and analysed, in duplicate, for bacteriophage number. The Petri dishes were kept in dark immediately after spread and during the incubation to avoid the inactivation of the bacterial host by the photosensitizer. Viral density (PFU mL^{-1}) was determined at each time of sampling as the mean of the two duplicates in the most convenient dilution series. Viral reduction at each time was determined by subtracting the mean number of phage surviving at each time by the number of phages at time zero and expressed as a $\log_{10}$ values. For each hybrid material two experiments were performed and the results presented are the average of the two assays.

3. Results

The non-immobilized photosensitizer used in this study inactivated the sewage T4-like phage to the limits of detection (>99.9999% of inactivation) with reductions of 7 log at the highest studied concentration (5.0 μM), after 180 minutes of irradiation. At 1.0 μM, a reduction of 3.6 log (99.92% of inactivation) was observed after 270 minutes. At the lowest concentration (0.5 μM), the rate of inactivation decreased to 93.74% (reductions of 1.8 log) after 270 minutes of irradiation (Figure 1).

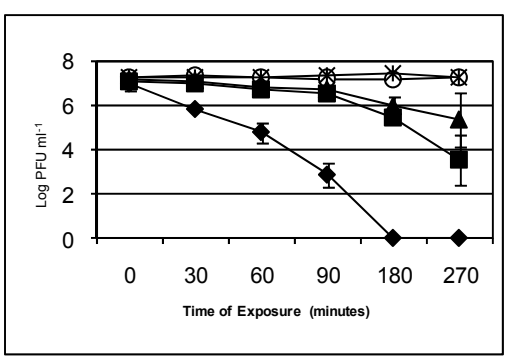

Figure 1. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of non-immobilized photosensitizer. (—o— light control, —✳— dark control, —●— 0.5 μM, —△— 1.0 μM, —▲— 5.0 μM).

The efficiency of the sewage bacteriophage inactivation by solid matrixes with immobilized porphyrins depends on the photosensitizer concentration and on the irradiation time. After 270 minutes of irradiation, Material 1 inactivated the sewage T4-like phage to the limits of detection for all the tested concentrations of photosensitizer (reduction of 6.9 log). For concentrations of 20 and 100 μM of photosensitizer reductions of 6.8 log and 6.4 log, respectively were observed after 60 minutes of irradiation (Figure 2). Material 2 show, after 270 minutes of irradiation, rates of inactivation ranging from 99.54 to 99.9999% with reductions of 2.3 log, 6.9 log and 4.5 log, for 5.0, 20 and 100 μM, respectively (Figure 3). The rate of phage photoinactivation with Material 3 after 270 minutes ranging from 99.76% to 99.98%, with reductions of 2.6 log, for 20 μM, and 5.2 log, for 100 μM (Figures 4).

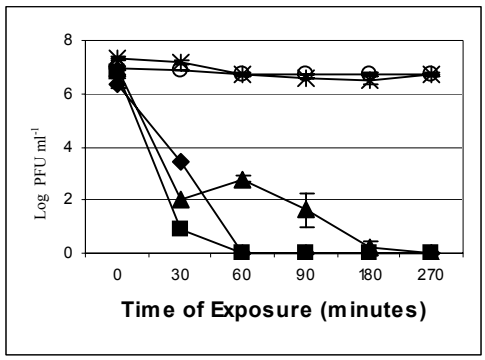

Figure 2. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of Material 1. (—o— light control, —✳— dark control, —▲— 5 μM, —■— 20 μM, —♦— 100 μM).

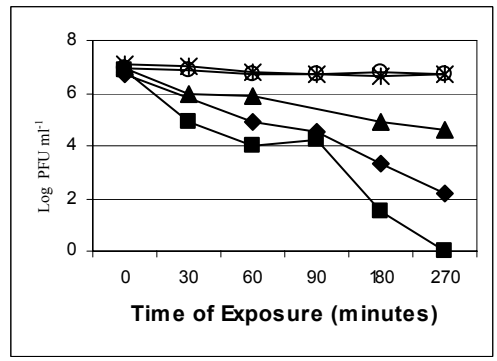

Figure 3. Density variation of the sewage bacteriophage after 30, 60, 90, 180 and 270 minutes of irradiation with 40 W m^{-2}, in the presence of Material 2 (—o— light control, —✳— dark control, —▲— 5 μM, —■— 20 μM, —♦— 100 μM).

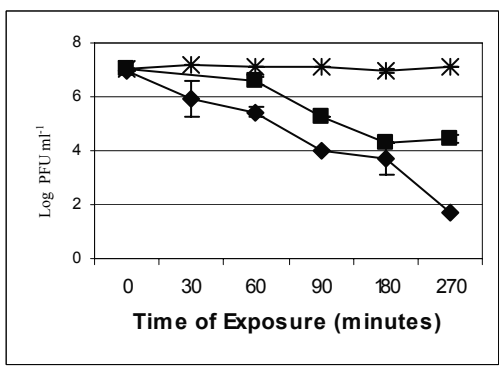

Figure 4. Density variation of the sewage bacteriophage after 30, 60, 90 and 180 minutes of irradiation with 40 W m^{-2}, in the presence of porphyrin Material 3. (—✳— dark control, —■— 20 μM, —♦— 100 μM).

4. Discussion

The results of this study show that the insoluble materials tested in this work, when irradiated with white light (40 W m^{-2}), can efficiently photoinactivate a sewage non-enveloped virus. The rate and the extension of inactivation are dependent on the material composition and on the photosensitizer concentration. In some cases immobilized photosensitizers in solid supports (Material 1) gave similar bacteriophage inactivation to the one observed with the non-immobilized photosensitizer at 5.0 μM.

The phage inactivation to the limits of detection (reductions of about 7 log) with Material 1, means that this insoluble material can be applied with the same efficacy of the non-immobilized photosensitizer in wastewater disinfection.

In conclusion, our results show that insoluble materials containing immobilized porphyrins, when irradiated with white light, efficiently photoinactivate an environmental T4-like phage, and open the possibility of using this new environmental friendly technology for wastewater disinfection. The use of immobilized photosensitizers avoids any residual traces of sensitizer in the water output. Besides this, the re-utilization of these materials considerably reduces the costs of photodynamic treatment and makes it a simple and cheap technology for wastewater treatment. The complete eradication of viruses with low light intensity means that this technology can be used throughout the year. Consequently, photoinactivation can be applied to wastewater disinfection under natural irradiation conditions.

Acknowledgements Thanks are due to the University of Aveiro, Fundação para a Ciência e a Tecnologia and FEDER for funding the Organic Chemistry Research Unit and the project POCI/CTM/58183/2004 and to CESAM (Centro de Estudos do Ambiente e do Mar) for funding the Microbiology Research Group. C. Carvalho and J. Tomé are also grateful to FCT for their grants (SFRH/BPD/5630/2001).

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The DGGE technique and 16S rDNA clone libraries analysis as a microbiological indicator of soil degradation

E. A. K. Rafedzi[1], M. Krsek[2] and E. M. H. Wellington[2]

[1] Strategic Resource Research Centre,Malaysian Agriculture Research and Development Institute, 50774 Kuala Lumpur, Malaysia.
[2] Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom.

In this experiment, the chosen site Basilicata in Italy consists of two types of soil treatments; bioagriculture (BA) and conventional agricultural (CA). The aim for this study was to determine the microbial diversity which can be use to differentiates the level of soil degradation and yet be the microbiological indicator. The soil samples were extracted for the DNA by combination of two conventional methods of Calvo-Bado *et al.* (2003) and Griffiths *et al.* (2000). The soil DNA was amplified for the target 16S rRNA genes using the Universal primer; P1 and P2. The amplified DNA was then run on the denaturing gradient gel electrophoresis (DGGE) to separate the PCR band. Marker was taken from the amplified soil DNA which produces a very strong band from one of the treatment. Two 16S rDNA clone libraries were constructed from the plot; BA and CA. Actinobacteria was the known genera that 26% and 38% dominate in BA and CA respectively. The specific results for Actinobacteria in clone libraries suggested that water content is highly correlated with actinobacteria numbers and diversity.

Keywords soil; microbial diversity; DGGE; clone library; Actinobacteria

1. Introduction

Soil is an important natural resource that can be easily disturbed and need to be preserved. Many properties must be used to define soil quality; chemical and physical soil parameters such as organic matter, nutrient content have been used to measure the soil quality (Parr and Papendick, 1997). But these parameters change very slowly and needs many years to measure the significant changes. Therefore, the soil biological and biochemical properties providing accurate information on soil changes. Soil microbial activity has a direct influence in an ecosystem stability and fertility (Smith and Papendick, 1993). Soil organism and biotic parameters, such as abundance, diversity, food web structure, or community stability meet most of the criteria for useful indicators of soil quality (Bunning, S. and Jimenez, J. J 2003). They respond sensitively to land management practices and climate and correlate well with beneficial soil functions, including water content and organic matter. Most of the microbial diversity studies conducted in complex ecosystem, such as soil have been biased essentially by the unculturability of many microorganisms and the lack of sensitivity of traditional microbiological methods (Hugenholtz et al., 1998).

The culture-independent approach has enabled us to obtain abundant information about the structures of microbial communities that had been previously explored only by culture-dependent strategies (Amann et al., 1995). In microbial ecological studies, however, it is important to understand the relationship between the structures and functions of microbial communities (Torsvik, 2002 and Wellington et al., 2003). Denaturing gradient gel electrophoresis (DGGE) are the most used for characterizing bacterial communities in environments ((Heuer et al., 2001). By using the DGGE, 50% of sequence variants can be detected in DNA fragment up to 500 bp. The percentage of the DNA fragment can be increased 100% if a GC rich sequence (GC-clamp) was added to one side of the DNA fragment (Myers et al., 1987). The use of GC-clamp in DGGE is very important because it has a high melting point which can prevent two DNA strands from complete dissociation into single strands (Muyzer et al., 1997). The 16S rRNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. Initially, the 16S rRNA was too large for phylogenetic analysis. However, the development of DNA cloning and sequencing protocols has facilitated the sequencing of the full 16S gene, which has become important for the analysis of microbial diversity as well as for phylogenetic studies (Olsen et al. 1986).

2. Materials and methods

2.1 DNA extraction, amplification and DGGE fingerprinting

All soil samples were stored at -20˚C. The soil DNA extraction was based on the established method of Griffiths et al., (2000) and Calvo-Bado et al., (2003). 0.5 g of soil sample was weighed in the ribolyzer tube

(BIO 101 System) and was ribolyzed for 30 seconds. This extraction process involved a phenol:chloroform:isoamyl solution. The top layer in the tube was extracted and transferred into microcentrifuge tube. A chloroform:isoamyl alcohol mixture was added to form a two layer of liquid. The mixture was mixed and the top layer was pipetted into another microcentrifuge tube and added with 2 volume of PEG solution. After 2 hours at room temperature the supernatant from the tube was poured off and the pellet washed with 70% ethanol. Then the pellet was suspended in 50 µl of TE buffer and stored at 4˚C.

A set of universal 16S primers were used to amplified part of the 16S rRNA gene: P1 5'-CCTACGGGAGGCAGCAG-3' and P2 5'-ATTACCGCGGCTGCTGG-3' (Muyzer, 1993). The samples were amplified as follows: 35 cycles of 94˚C for 1 min, 64˚C for 1 min and 72˚C for 2 min with a final extension at 72˚C for 10 min. After amplification, the PCR products were loaded on 10% (w/v) polyacrylamide gel in 1x TAE buffer. The denaturing gradient ranging between 40% to 75%. The electrophoresis was run at 60V for 18 hours at 60˚C. DGGE gels were stained with SyberGreen Nucleic Acid I and analysed using GeneTools and GeneDirectory software by SYNGENE.

2.2 Cloning

Clone libraries have been generated to determine the dominant genera in Basilicata site. Cloning was done by using the TOPO TA cloning kit (Invitrogen) following the manufacturers' recommendations.

3. Results and discussions

3.1 Denaturing gradient gel analysis (DGGE)

From the DGGE image shown, the branch separated between bioagricultural (BA) and conventional agricultural (CA) can be distinguished clearly. But there were replicates (from BA; track 2 and from CA; tracks 7 and 13) from both treatments which separated from their group. So, the soil in this site can be categorized as a heterogeneous soil. These results indicate that the dominant microbial genera were not much affected by the application of either fertilizer or pesticide application in this soil. The conventional and bioagricultural practices appeared to have an impact on microbial diversity. The banding pattern in Basilicata showed that the diversity within treatments was quite heterogeneous and therefore no conclusions could be drawn.

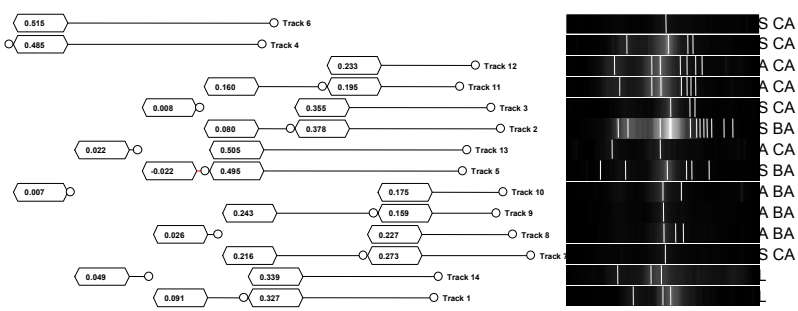


Fig. 1 The dendogram of DGGE image of Basilicata site analyzed by Gene Tool software.

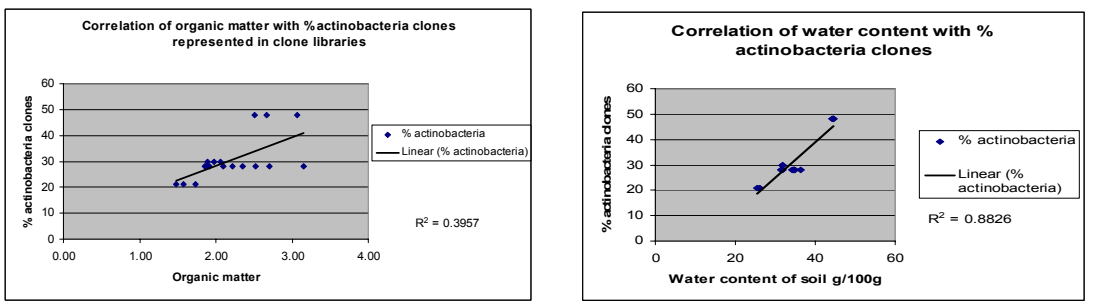
*Key: CA- conventional agricultural, BA – bioagricultural

3.2 Clone library analysis

A total of thirty nine clones (data not shown) obtained from biological agriculture (BA) site at Basilicata. Sequence analysis of the BA site revealed that ten clones were Actinobacteria. In conventional agriculture (CA) site, actinobacteria comprised 38% (13 clones) of total clones. *Jiangella gansuensis*, *Nocardia plantarum* and *Frankia sp*. were known actinobacteria clones and the remaining were uncultured. *α-Proteobacteria* was the only proteobacteria subdivision identified in CA (figure 3). When comparing the relative abundance in both two sites at Basilicata, Actinobacteria were more abundant in the conventional agriculture site rather than in the biological agriculture site where as the opposite was true for the proteobacteria. The uncultured bacteria consisted of nitrogen cycle bacteria and other bacteria such as an uncultured *Clostridium, Staphylacoccus sp.,* and *Thermomicrobium roseum.*

3.3 Correlation analysis of the actinobacteria with the organic matter and water content

The correlation analysis was carried out to observe the significance of the dominant genera in both sampling site, the Actinobacteria with the represent parameter. When the soil condition was in the low water content which means the soil structure are not capable to retain the water content then the organic matter in that soil should be low. This is because the activity of the microorganisms in that particular soil are slow with the decreased of the water content and the results of their activity was degradation process which also can be slow. There is a positive relation between the % organic matter and the % of actinobacteria clones. The trend showed that when organic matter increased, there was also an increase in the % of actinobacteria clones. The relationship between the water content and % of actinobacteria is linear. The trend is positive. The % of actinobacteria increased when with increasing water content.



(a) (b)

Fig. 2 a) Correlation of % actinobacteria clones represented in clone libraries with organic matter content (P=0.005) and b) correlation of % actinobacteria clones represented in clone libraries with water content ($P=7.5x10^{-9}$).

3.4 General discussions

The DGGE gels were analysed with the Gene Tool and Gene Directory (SYNGENE) software that construct dendograms based on similarities in banding patterns. DGGE banding patterns did not cluster with treatment in some cases when dendrograms were construted from banding patterns. The conventional and the bioagricultural practices appeared to have an impact on microbial diversity. Basilicata plot which had a bioagricultural site and conventional agricultural were used to investigate whether pesticide and fertilizer usage with the natural compost like green manure affected the diversity of soil microorganisms. Microbial activity is a term used to indicate the vast range of activities carried out by microorganisms in soil, whereas biological activity reflects not only microbial activities but also the activities of other organisms in the soil, including plant roots (Nannipieri et al., 1990). When the soil activity had been influenced by any mechanism involved in soil, the microbial diversity also can be affected (Gelsomino et al., 1999).

Phylogenetic tree for selected site in this experiment give an accurate results on the abundance genera identified at each site. Actinobacteria are most commonly found in soil and play an important role in decomposition of organic matter. The results based on 16S rDNA library did not indicate a difference in bacterial diversity between the different agriculture treatments but the abundant genera showed a different percentage in different treatments. The use of molecular approach and fingerprinting profiling indicated that organic matter content was the most important factor to determine the level of the microbial diversity in degraded soil. It can be concluded that soil degradation had an impact on the soil microbial diversity and bacterial numbers in soil. The diversity of the soil microorganisms was dependent on the treatments such as organic matter amendment, plant species and plant cover which affected soil structure and function.

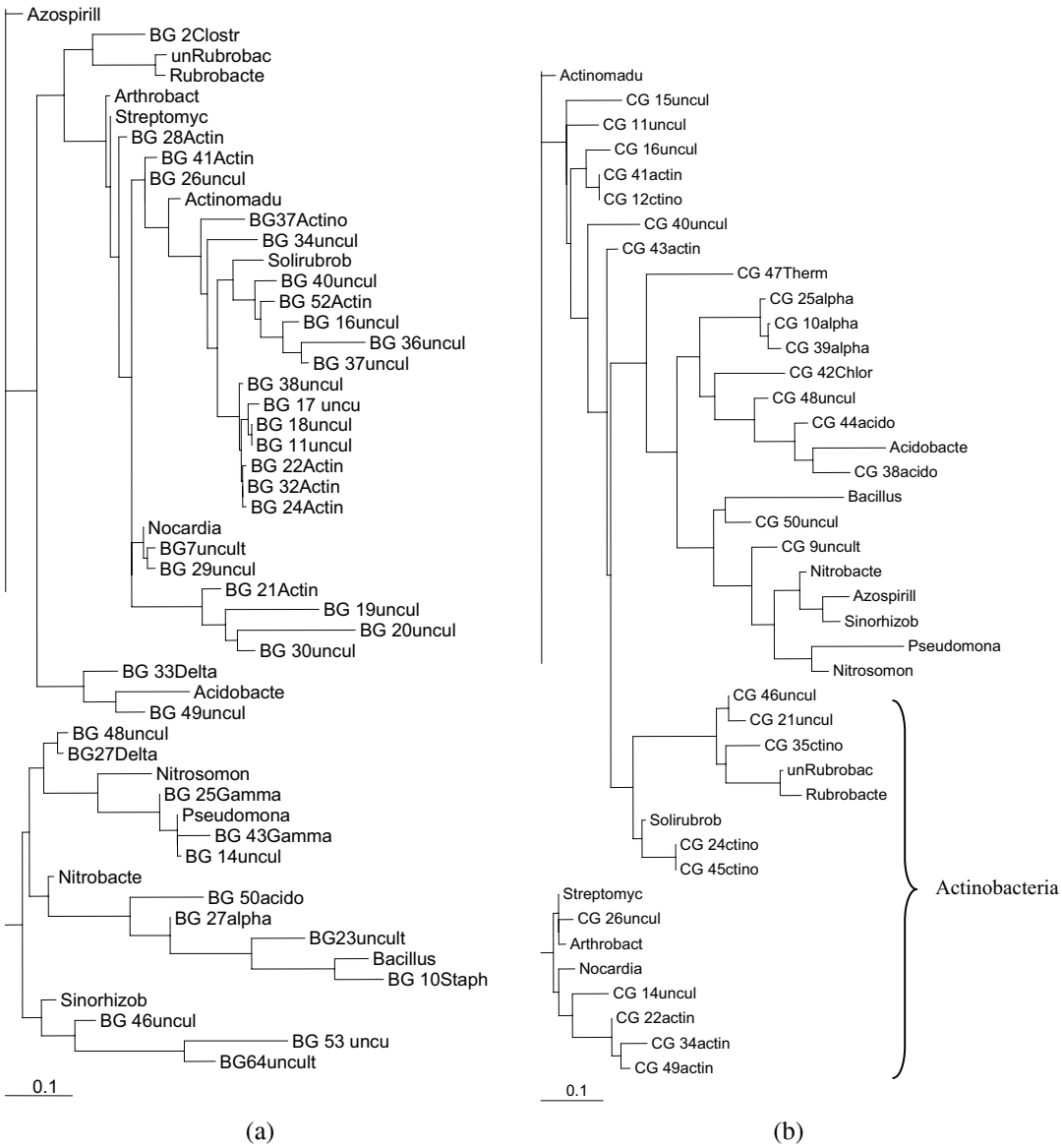


Fig. 3 a) Phylogenetic tree of Basilicata (bioagriculture) and b) phylogenetic tree of Basilicata (conventional agriculture) containing partial 16S clone sequence.

Acknowledgements This study was conducted during the MSc study. The support by Prof Elizabeth Wellington as a supervisor and Dr Martin Krsek as a lab supervisor were much appreciated and gratefully acknowledged.

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The Testate lobose amoebae in the wastewater treatment

A. C. Tomasini Ortiz[1], G. E. Moeller Chávez[1], M. Garzón-Zuñiga[1] and Y. Hornelas Orozco[2]

[1] Mexican Institute of Water Technology (IMTA). Jiutepec, Mor. Mexico. atomasini@tlaloc.imta.mx, gmoeller@tlaloc.imta.mx, mgarzon@tlaloc.imta.mx

[2] Institute of Marine Sciences and Limnology (ICMyL). National Autonomous University of Mexico. Mexico City.

The ecology of municipal and industrial wastewater treatment is complex biocenoses. In fixed film systems or in suspended growth systems; besides, the bacterial consortium, several protozoan genera have been reported: testate amoebae, or organisms with a shell. Amoebae are commonly found in activated sludge systems. But just one or two genera have been reported. This study describes the protozoan ecology found in wastewater treatment systems with suspended and fixed biomass, fed with municipal and industrial wastewater (textile industry, dye process).
Amoebae are found various shapes and sizes (10 to 200 µm). Movement is due to cilia, pseudopodia or false feet, most of them reproduce asexually by cellular fission; they grow over organic particulate matter and tolerate low dissolve oxygen concentration. Amoebae are commonly found in treatment systems with nitrification and low organic load. They commonly eat bacteria, phytoplankton, particulate organic matter, cellulose and lignin. They are known as *"panphytophagous"*, because they eat both detritus and living organisms. These characteristics allow these genera to treat water.
The most common genera found in biological wastewater treatment are: *Arcella, Euglypha*, *Centropyxis, Trinema, Bullinularia* and *Diflugia*.

Keywords Amoebae, Water treatment organisms, Wastewater

1. Introduction

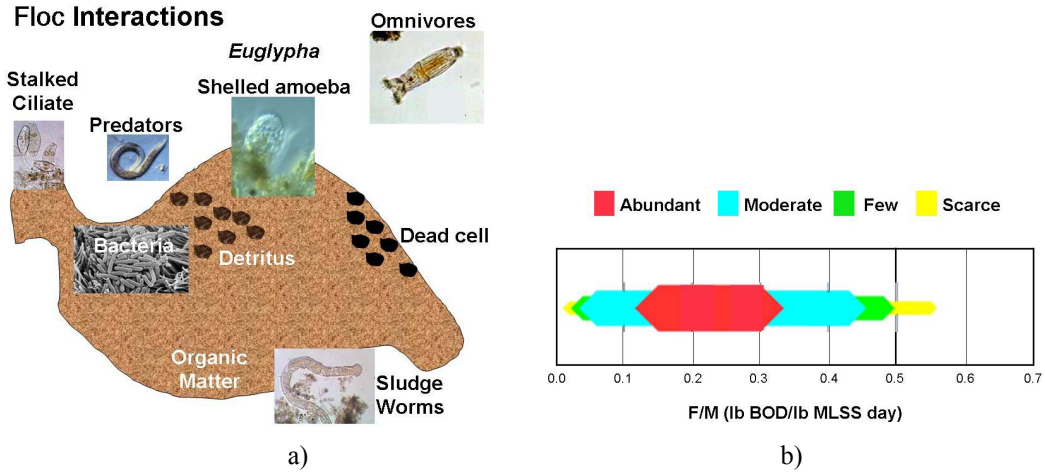
The ecology of municipal and industrial wastewater treatment is complex biocenoses. Either in fixed film systems or in suspended growth systems, besides the bacterial consortium, there has been reported several protozoan genera, the testate amoebae or amoebae with shell, had been reported as an important faunistic part of activated sludge. But in this type sludge, the specialized literature mentions only one or two genera, but, by contrast, we had found a great variety of genera and species.

The testate amoebae are a polyphyletic group of protozoan that belongs to the rhizopoda. They distinguish from naked amoebae in their ability to form an outer shell. This shell has an opening through which the pseudopodia extend, referred to as "pseudostome". The shell length of the testate amoebae ranges between 15 and 170 µm. The more common species tend to be smaller than 45 µm. They live in soil pores filled with water and within the thin water-film around detritus of soil particles (Schröter, 2001).

Amoebae move with the help of cilia, "pseudopodia" or false feet, most of them reproduce asexually by cellular fission; they grow over organic particulate matter and tolerate low dissolve oxygen concentration. Amoebae are commonly found in treatment systems with nitrification and low organic load. They commonly eat bacteria, phytoplankton, particulate organic matter, cellulose and lignin; this strategy is known as *"panphytophagous"* (Luxton, 1972), because they eat both detritus and living organisms. These characteristics allow these genera to treat water (López-Sáez and López-Merino, 2005).

The most common genera found in the sludge of biological wastewater treatment are mainly the *Euglypha* genera as an important group during the microscope search. This testate amoebae presents some interaction with the floc, as it is shown in figure 1a; the frequency and occurrence is presented in figure 1b.

Testate amoebae are common in soils, treatment plants and in creek bottoms. They are adapted to a wide range of environment conditions and, therefore, they are good index organisms, as in the following situations: effluents with a BOD of 0 to 50 mg/L and effluents with ammonium of 0 to 30 mg/L.



a) b)

Figure 1. (a) Floc interactions, *Euglypha sp*., (b) Range of occurrence and relative abundance *Euglypha sp*.

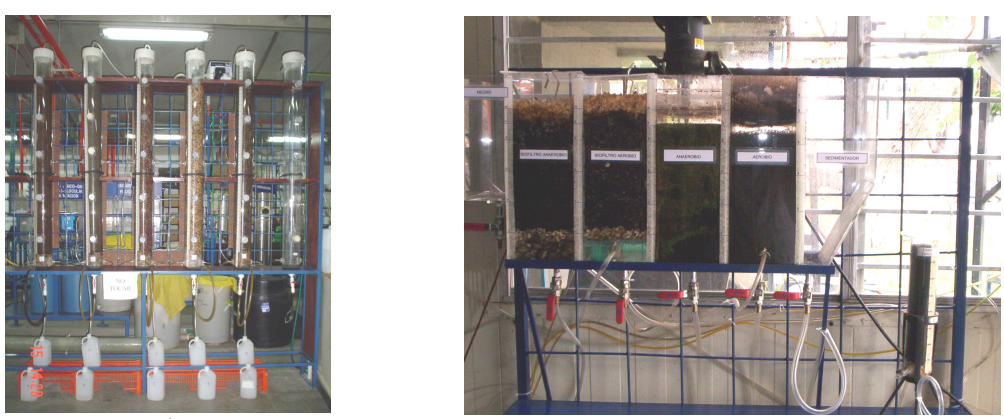
2. Methodology

Six reactors of fixed biomass were mounted and packed with different vegetable materials ("Tabachín" and "Jacaranda" are local tropical trees), see table 1.

Table 1. Packing materials of the six fixed biomass reactors

Biofilter code	Packing materials
BF1	Tabachín + coconut fiber
BFT	Turf + wood pieces
BFA	Sugar-cane bagasse + Jacaranda
BFB	coconut fiber + Jacaranda
BFC	Sugar-cane bagasse + Tabachín
BFD	coconut fiber + Tabachín

BF1 and BFT biofilters were added with dye wastewater and BFA, BFB, BFC and BFD, were feeder with municipal raw wastewater. All the reactors were aerobic (figure 2a). By the other side, there were aerobic and anaerobic fixed biomass biofilters, which were feeder with wastewater plus type azo dyes (figure 2b).



a) b)

Figure 2. (a) Fixed biomass packed biofilters. (b) Suspended biomass biofilters.

Samples were taken from the packing materials and from the sludge of the biofilters of suspended biomass in both anaerobic and aerobic conditions, this were observed a) anaerobic condition in a scanning electron microscope (SEM) JEOL, model JSM – 6360 LV; and b) aerobic condition, in a Olympus optic microscope, model BX60.

3. Results

SEM observations of the parking media showed a great abundance of *Euglypha rotunda* (figures 3a & 3b), followed by *Trynema enchelys* (figures 3c & 3d), *Bullinularia indica* (figure 3e), *Diflugia gutula* (figure 3f), *Arcella discoides* (figure 3g) and *Bullinularia sp* (3h). There was a slight or null presence of bacteria, which confirms that the testate amoebae were feeding with them.

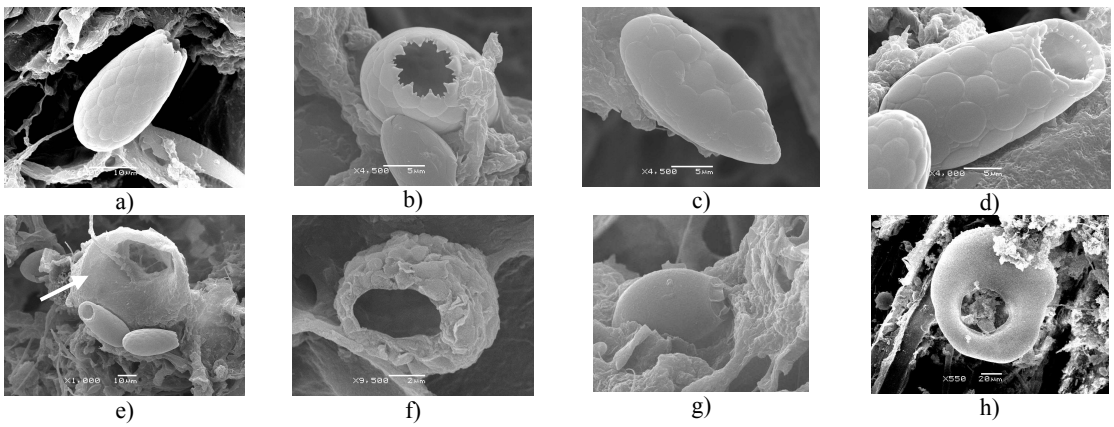


Figure 3. a) Euphlypha rotunda, b) View of the mouth or pseudostome of Euglypha rotunda, c) Trynema enchelys, d) View of the pseudostome of Trynema enchelys, e) Bullinularia indica, f) Diflugia gutula, g) Arcella discordes, h) Bullinularia sp.

The observation from the sludge of the biofilters of suspended biomass in anaerobic and aerobic conditions, were: In the case of the aerobic biofilters, besides the normal components of the activated sludge, like: Ciliates, Rotifers, etc., it was found the presence of testate amoebae such as, in abundance order: *Euglypha rotunda* (figure 4a), *Euglypha acanthophora* (figure 4b), *Centropyxis spp.,* (figures 4c, 4d & 4e) and Arcella sp. (figures 4f, 4g & 4h).

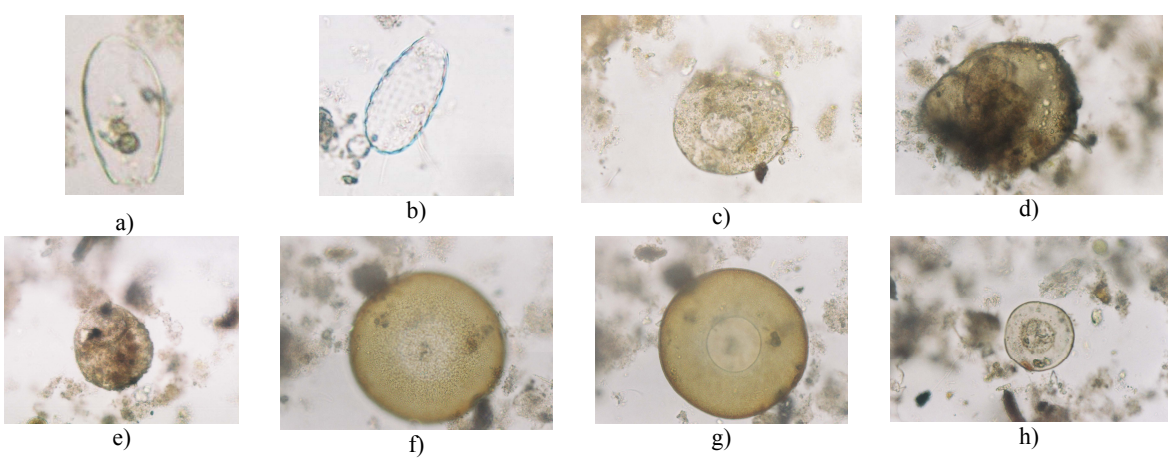


Figure 4. a) Euphlygia rotunda, b) Euglypha acanthophora, c) Centropyxis sp, d) Centropyxis sp, e) Centropyxis sp, f) Arcella sp, g) Arcella sp, h) Arcella sp.

Nevertheless, the anaerobic biofilter just show –in great abundance– various species of *Arcella sp* (figures 5a – 5f) *Centropyxis spp* (figures 5g – 5i) and *Lesquereusia gibbosa* (figures 5j – 5l).

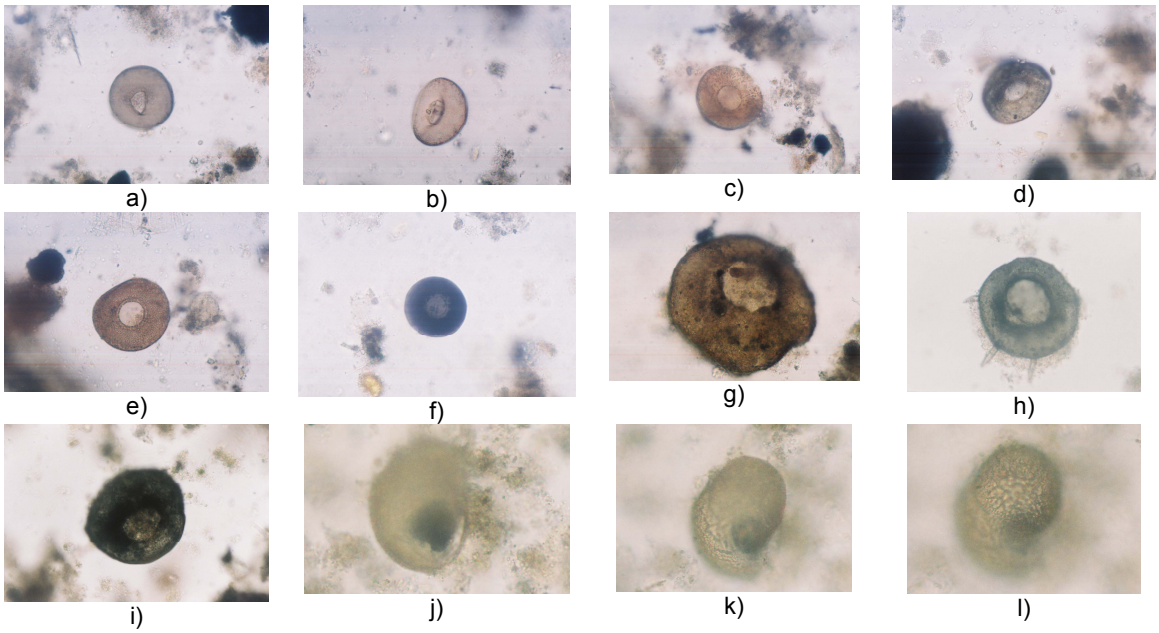


Figure 5. a) Arcella sp, b) Arcella sp, c) Arcella sp, d) Arcella sp, e) Arcella sp, f) Arcella sp, g) Centropyxis sp, h) Centropyxis sp, i) Centropyxis sp, j) Lesquereusia gibbosa, k) Lesquereusia gibosa, l) Lesquereusia gibosa.

A microscope count were performed of the sludge present in the aerobic suspended biomass reactor, and with the idea of the organisms that normally are present in this media, there were less bacteria, with the percentage result presented in figure 6. It can be appreciated that the percentage of presence of ciliates and testate amoebae were the same and it is high in comparison with the one of the rotifers and the algae.

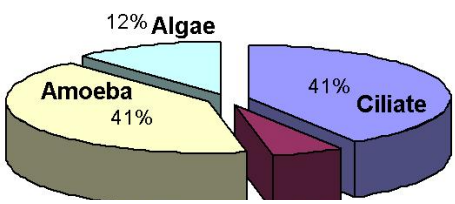


Figure 6. Microorganisms presence percentage in activated sludge.

In the activated sludge literature reports a relative growth of the microfauna and flora as it can be seen in figure 7, in which it can be appreciated that the presence of the testate amoebae is not taken into account and that the naked amoebae are present in low proportions, an at the very beginning of the sludge stabilization.

It also was observed that in the fixed packed biomass reactors, the nitrogen removal, phosphorous and fecal coliformes were excellent, most of all of the coliformes, because they were ultimately of cero fecal coliformes MPN/100 mL. This suggests that the main foods of the amoebae are bacteria and nutrients.

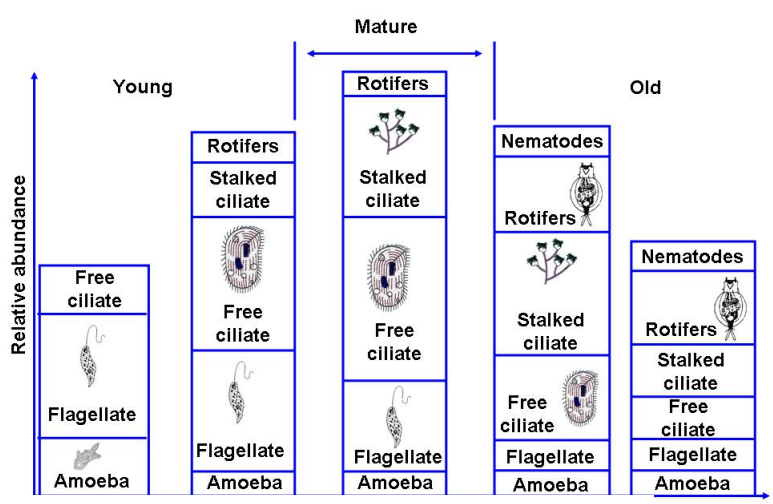


Figure 7. Microorganisms relative growth in the course of activated sludge stabilization.

4. Conclusions

It can be seen that *Arcella spp.*, *Centropyxis sp., and Euglypha spp.*, are found both in aerobic and anaerobic biological systems.

The genera of the testate amoebae more frequently founded in the biological treatment of wastewater are: In anaerobic environments, *Centropyxis spp.*, and *Lesquereusia gibbosa,* and in aerobic environments, *Euglypha spp.*, *Arcella spp.*, *Trynema sp.*, *Bullinularia* spp., *Centropyxis spp.*, and *Diflugia spp.*

It would be worth to review more carefully and to follow in a closer manner, the presence and function of these amoebae in the biological reactors, both in anaerobic and aerobic conditions, in relation with the treatment of wastewater.

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Treatment of linear alkylbenzene sulfonate in mesophilic anaerobic sequencing batch reactor

I. C. S. Duarte*, L. L. Oliveira, N. K. Saavedra, D. Y. Okada, M. S. Mayor, and M. B. A. Varesche

Department of Hydraulics and Sanitation, School of Engineering of São Carlos, University of São Paulo, Av. Trabalhador Sãocarlense, 400, Zip Code 13566- 590, São Carlos-SP, Brazil.

*Corresponding author: e-mail: iolanda.duarte@gmail.com, Phone:+55 16 3373 5387

The aim of this search was to assess the linear alkylbenzene sulfonate (LAS) degradation in an anaerobic sequencing batch reactor (ASBR). It was added domestic liquid detergent in the concentration of the 22 mgLAS/L. At the end of operation (143 days) it was obtained 53% of degradation of the total mass of LAS adsorbed and only 1.5% was adsorbed in the biomass. In the last phase, the sludge digestion was promoted and the adsorbed LAS mass was degraded in 96%. It was noted that the methanogenic archaea were presented in all phases by PCR/DGGE technique, with affiliation: *Methanosaeta* (98-99%), *Methanospirillum* (90%), and *Methanobacterium* (96%). The microorganisms of the *Bacteria* Domain presented affiliation with *Opitutus* (96%) and *Arcanobacterium* (94%). The LAS degradation was larger in the absence of more organic sources and the bacteria developed in this stage used the molecule of the surfactant as carbon and energy sources.

Keywords anaerobic sequencing batch reactor, linear alkylbenzene sulfonate, PCR/DGGE, 16S rRNA

1. Introduction

Linear alkylbenzene sulfonate (LAS) is present in the sanitary sewage and some industrial wastewater. This detergent is often not degraded in conventional treatment, causing adverse effects on aquatic ecosystems. Despite of being found in low concentrations in the wastewater, the anionic surfactants can be adsorbed in the biological sludge and in sediments.

Among the different configurations of anaerobic bioreactors used in LAS treatment, the most applied is UASB (Upflow Anaerobic Sludge Blanket) in bench scale [1], [2] and [3]. Researches of anaerobic degradation were developed in Brazil at the Laboratory of Biological Processes - EESC/USP using horizontal anaerobic immobilized biomass (HAIB) reactor with different inoculums and different support media as, for instance, polyurethane foams, charcoal and expanded clay for immobilization of the biomass. At the moment other configurations are being appraised to reach higher degradation's levels.

Thus, the aim of this work was to evaluate the microbial population present in linear alkylbenzene sulfonate degradation in anaerobic sludge biomass reactor operated in sequencing batch.

2. Material and Methods

The ASBR reactor was a glass cylinder (total volume = 5L), operated in sequencing batch. The reactor was operated in cycles of 24 hours, being 15 minutes of stuffing, 23 hours of reaction, 30 minutes for sedimentation and 15 minutes for discharge. The system was maintained in acclimatized camera at 30ºC and rotation was 50 rpm. The reactor was fed with synthetic substrate (3 L). The media COD was 180 mg/L, referring of organics sources (sucrose, yeast extract and starch).

The ASBR was inoculated with anaerobic sludge from UASB reactor treating swine wastewater (Universidade Estadual Paulista, Campus Jaboticabal-SP, Brazil).

Five experimental phases were defined by changes in to compose the synthetic substrate (Table1).

Table 1 Phases of ASBR operation

Phases	Period (days)	Characteristics
I	1 a 21	Feeding with synthetic substrate
II	22 a 77	Addition of LAS (22 mg/L)
III	78 a 91	Increase in concentration of yeast extract
IV	92 a 143	Feeding with LAS (only) 22 mg/L LAS
Sludge digestion	144 a 168	Absent feeding

Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) levels were determined according to the procedures described in the [4].

Detergent degradation was measured by the LAS concentration by HPLC method [5].

The solid samples (duplicate) were extracted with methanol in ultrasound bath for 30 minutes to measure the adsorbed and precipitated LAS present in the biomass. Then, the methanol samples were filtered and analyzed by HLPC. This protocol of extraction presented 85% of efficiency.

The microbial diversity present in ASBR was determined using the PCR/DGGE technique. The biomass samples were obtained at the end of each phase. The total DNA was extracted using the protocol described by [6].

For the DGGE analysis, 16S rRNA gene fragments were amplified by PCR using specific *primers*: 1100F and1400R for *Archaea* Domain [7], 968F and1392R for *Bacteria* Domain [8]. A GC-clamp [9] was added to the forward *primers* of the two primer sets. A DNA template of 2.0 µl was added to the amplification reaction, by the instructions of the supplier manual for Taq DNA polymerase platinum (Invitrogen®).

The amplified DNA fragments were separated by Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was conducted using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercoles, CA). PCR products were applied onto 8% (wt/v) poliacrylamide gel in 0.5 x TAE, with the linear gradient of denaturants (urea and formamide) ranging from 30% to 60% for *Bacteria* Domain and 40% to 60% for *Archaea* Domain. Electrophoresis was performed at constant voltage of 75V and temperature of 65ºC during 16 hours. Gels were observed in UV illumination and photographed using the equipment Eagle Eye II (Stratagene).

Some bands excised the DGGE gel was amplified for PCR using same *primers* sets but without GC clamp. PCR products were sequenced using BigDye™ terminator cycle sequencing version 3.0 sequencing kit according to the manufactures's instructions (Applied Biosystem) and sequenced using ABI Prism 310 (Applied Biosystem). The primer 968F and 1100F were used in the sequencing reaction.

The sequences were verified using the DNAStar software program and compared to sequences deposited in GenBank by performing a BLAST search.

3. Results and Discussion

The ASBR reactor was fed during 21 days with synthetic substrate without LAS. In this first experimental phase the organic matter removal efficiency was 74%. However, the concentration of measured biomass in volatile solids passed of 43.1 g/L to 6.2 g/L. This decrease in the concentration biomass was attributed a low concentration of the organic matter in the influent (149±83 mgCOD/L).

After this period, 22 mg/L of LAS was added to the influent during 50 days (phase II). In this period, the VS concentration passed of 6.2 g/L to 2.2 g/L. This decrease of biomass was attributed to the presence of LAS, once the VS in effluent were not significant.

LAS was monitored in liquid phase and was verified that soon after LAS addition, almost all the detergent was adsorbed in biomass. The LAS presence also effected the COD removal (44±14%).

In 50 days 5.39 g of LAS were added, 2.94 g were recovered in effluent and 0.441 g was adsorbed or precipitant in biomass, resulting in LAS degradation of 37%.

In phase III were added 500 mg/L of yeast extract to favor the cellular growth. The COD passed of 183±35 mg/L to 771 mg/L of COD. However, such procedure was not positive, because the COD removal decreased reaching 28%.

For the calculation of LAS degradation efficiency, was adopted the accumulated LAS mass along the operation. It this way, even 91 days, 6.93 g of LAS were added, 3.88 g were recovered in effluent and 1.34 g were adsorbed or precipitant in biomass, resulting in 24.5% of degradation.

The excess nutrients caused decreased in LAS degradation efficiency. The co-substrates were utilized by microorganisms. The feeding in phase IV, consisted only by detergent (22 mg/L of LAS).

The mass of LAS added up to 143 days was of 13.1 g, 5.9 g were recovered in the effluent and only 0.21 g was adsorbed in biomass. The mass of LAS adsorbed in biomass represented 1.6% of the total mass of LAS added, and then the degradation was 53.3%.

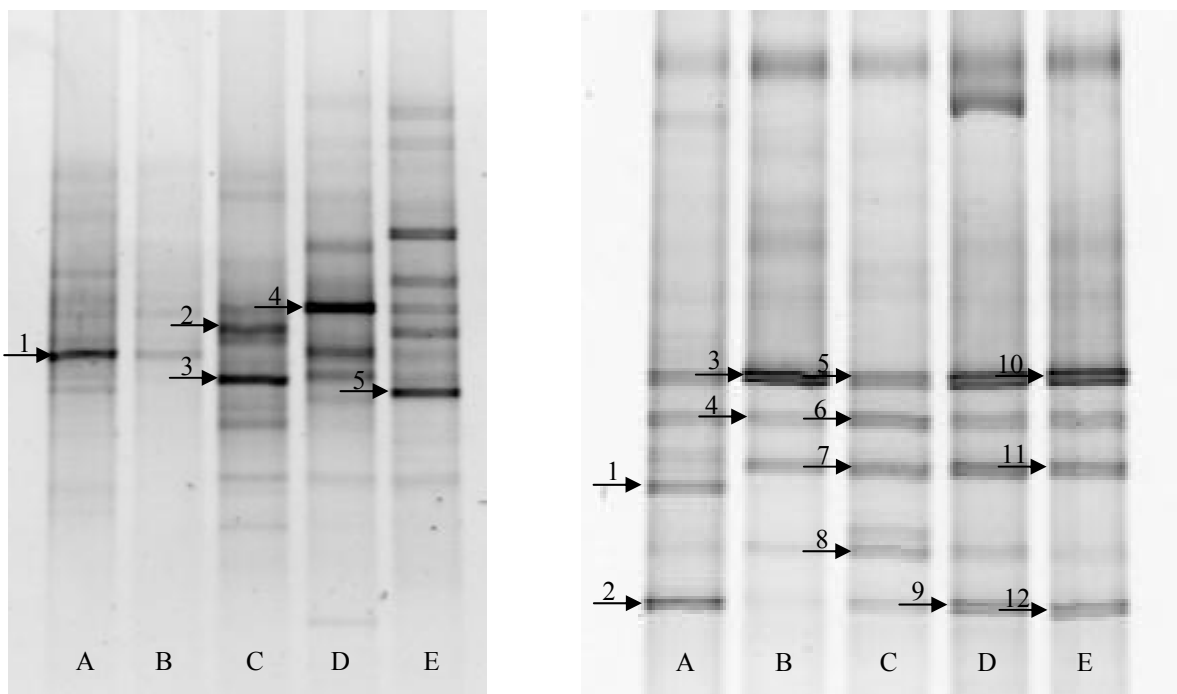
However, for getting of a treatment without residual LAS, the present biomass in the reactor (2.2 g/L of VS) it was submitted to the digestion. At the end of 21 days the degradation of LAS adsorbed reached 96% and the concentration of VS stayed the same.

The microbial community present in the reactor was analyzed by PCR/DGGE. It could be observed that the detergent addition brought significant changes to population of microorganisms of *Bacteria* Domain (Figure 1a) and same without co-substrates, there was great microbial diversity. However, some populations were favored as, for instance, the population represented by the band 2 and 5 that by the partial sequencing of the gene 16S RNAr presented affiliated with *Opitutus* sp (96%) and *Arcanobacterium* sp (94%) (Table 2).

Table 2 Phylogenetic affiliation of DGGE bands of *Bacteria* Domain

Bands	Accession number (Genbank)	% Similarity	Reference
1	Bacterium uncultured (EF602476)	96	[10]
2	*Arcanobacterium* (AJ250959)	94	[11]
3 and 4	Bacterium uncultured (AB240373)	96-99	[12]
5	*Opitutus* sp (X99392)	96	[13]

Even not being possible quantified the biogas, the DGGE gel (Figure 1b) proved the presence of methanogenic archaea. The detergent addition also influenced in the diversity of *Archaea* Domain, with decrease of population diversity soon after to add LAS. It was observed that, after to add LAS in the phase III, the microbial diversity was close the one of the inoculum. This showed that the inhibition LAS on the methanogenic microorganisms were temporary and reversible. It was also noticed that most of the populations of that Domain was affiliated with *Methanosaeta* sp. (99%) showed by bands 3, 5, 7 and 10, *Methanospirillum* sp with 90% (Band 1) and *Methanobacterium* with 89% showed by band 8.



(a) (b)

Fig. 1 DGGE gel illustrating the microbial community profiles (a) *Bacteria* Domain and (b) *Archaea* Domain. The numbers indicate of bands that sequences and the letters indicate the operation phases: (A) Inoculum; (B) Phase I; (C) Phase II; (D) Phase IV and (E) sludge digestion.

4. Conclusion

The mass balance showed that ASBR inoculated with anaerobic sludge from UASB reactor used in the swine wastewater treatment was capable to degrade LAS present in the used domestic detergent, with 53% of efficiency without of co-substrates.

Co-substrates of easy assimilation as sucrose, starch and yeast extract can cause decrease in the efficiency of degradation of LAS, once they are consumed preferentially.

The total mass of LAS applied in the reactor was 13.1 g, being 5.9 g recovered in the effluent and 0.2 g adsorbed in the biomass. During to digestion of the sludge, the LAS mass adsorbed was degraded in 96%. Even

in the feeding absence it was verified the presence of bacteria and methanogenic archaea in the biomass of the sludge. That permanence can be attributed to consume of LAS adsorbed and other organic sources remainders.

It was observed that LAS selected some bacterial populations and methanogenic archaea by analyzes of PCR/DGGE. Most of DGGE gel bands for the *Archaea* Domain presented affiliated with *Methanosaeta*.

Acknowledgements This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). The authors acknowledge the grants received from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ).

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Food Microbiology

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A simple method for simultaneously isolating mitDNA and virus dsRNA from wine yeasts

M. Maqueda Gil[1] and M. Ramírez Fernández[*,1]

[1] Departamento de Ciencias Biomédicas Área de Microbiología, Edificio Juan Remón Camacho, Avda. de Elvas s/n. 06071 Badajoz, Spain.

[*]Corresponding author: e-mail: mramirez@unex.es, Phone: +34 924289426, Fax: +34 924289427

A new method for the isolation of mitDNA and virus dsRNA is described for yeast characterization. It facilitates the ecological study of wine yeasts avoiding the necessity of using two different methods for the separate isolation of mitDNA and dsRNA. Also, it is faster and less costly than previous alternative methods, while giving similar nucleic acid yields.

Keywords mitDNA; wine yeast; killer phenotype; dsRNA virus

1. Introduction

Yeasts have numerous applications in modern and traditional biotechnology, such as production of food, unicellular protein, and products with added value. In the last few decades they have been incorporated into the biotechnology industry as hosts in the production of eukaryote proteins. They are also agents responsible for damaging fresh and prepared food. Therefore, it is interesting to be able to quickly and accurately identify yeasts that are significant industrially, environmentally, and clinically. Yeast taxonomy has been supported by conventional techniques, based on morphological and physiological descriptions of species and genera, but it depends on strain culture conditions which have led to errors in taxonomy and duality in their nomenclature. These problems have been resolved with the application of molecular techniques based on nucleic acid sequencing, karyotyping electrophoresis, microsatellite analysis, mitochondrial DNA length polymorphism, restriction fragment length polymorphism of ribosomal RNA, random amplified polymorphic DNA, and low and medium molecular weight RNA. The killer biotype has also been reported as a tool to evaluate yeasts [1,2]. We here describe a new method for the isolation of yeast mitDNA and virus dsRNA for yeast characterization. It facilitates the ecological study of wine yeasts by avoiding the need to use two different methods for the separate isolation of mitDNA and dsRNA.

2. Materials and Methods

2.1 Yeast strains and growth conditions

The origins of the *Saccharomyces* industrial strains used to validate the method are listed in **Table 1**. Yeast cells were grown 24 hours in 5 ml of YEPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose) with shaking at 30°C.

Table 1 *S. cerevisiae* strains used to validate the method.

Strain	RFLP mitDNA Pattern	Genotype	Origin
EX88	P88	*MATa/α HO/HO LA M2* [k2$^+$]	J. A. Regodón[a].
EX73	P73	*MATa/α HO/HO LA M2* [k2$^+$]	J. A. Regodón
EX1124	P198	*MATa/α HO/HO LA M2* [k2$^+$]	M. Maqueda[b].
EX1125	P41	*MATa/α HO/HO LA M2* [k2$^+$]	M. Maqueda

[a] J. A. Regodón, Departamento de Química Analítica, Universidad de Extremadura, Badajoz, Spain.
[b] M. Maqueda, Departamento de Ciencias Biomédicas Área de Microbiología, Universidad de Extremadura, Badajoz, Spain.

2.2 Nucleic acids isolation by yeast chemical breakdown

The method of Fried and Fink [3] for virus dsRNA isolation as modified by Sommer and Wickner [4] was followed (**Fig. 1B**). Cell cultures (5 ml) were spun down, washed with 50 mM Na_2EDTA (pH 7.0), spun down again, and incubated in 1 ml of 50 mM Tris-H_2SO_4, pH 9.3/2.5% 2-mercaptoethanol for 15 min at room

temperature. Next, cells were spun down, resuspended in 0.5 ml of 0.1 M NaCl/10 mM Tris-HCI, pH7.5/10 mM Na_2EDTA/0.2% sodium dodecyl sulfate (SDS). An equal volume of phenol (pH 8) was added, followed by incubation at room temperature for 30 min with shaking. Nucleic acids were recovered from the aqueous phase by ethanol precipitation (with 2× volume) after addition of 1/10 volume of 3M potassium acetate. The mix was incubated for 30 min at -20°C, centrifuged for 10 min in a microfuge (14 000 rpm, 1 min), washed with 70% (v/v) ethanol, recovered by centrifugation, vacuum-dried (2 min), and dissolved in 50µl of TE pH 8.

2.3 Nucleic acids isolation by yeast enzymatic breakdown

The simplified method of Querol et al. (2001) for mitochondrial DNA isolation [5] was followed (**Fig. 1C**). Cell cultures (1.5 ml) were spun down (2500g, 2 min), washed with sterile water and resuspended in 1 M sorbitol/0.1 M EDTA pH 7.5. After adding 20 mg/ml of Zimolyasa 60, the samples were incubated 20 min at 37°C. Spheroplast aliquots were centrifuged for 1 min in a microfuge (10 000 rpm, 1 min), and resuspended in 50 mM Tris–HCl pH 7.4, 20 mM EDTA, 10% w/v SDS, and the mix was incubated at 65°C for 5 min. Immediately, 0.04 ml of 5 M potassium acetate was added, and the tubes were placed on ice for 5 min. Then, they were centrifuged in a microfuge (14 000 rpm, 15 min) at 4°C. The supernatant was transferred to a fresh microfuge tube, the DNA was precipitated by adding 1 volume of previously cooled isopropanol, 5 min at room temperature, and centrifugation (10 min, in microfuge at 4000 rpm). The precipitated DNA was washed with 70% v/v ethanol, vacuum-dried, and dissolved in 16 µl of TE pH 8.

2.4 Digestion with restriction enzymes

The RNA was removed by RNA*ase* A (Sigma-Aldrich, USA) digestion, and mitDNA was digested with *Rsa*I according to the instructions of the supplier (Sigma-Aldrich, USA). The digestion was done in a mix containing 3µl of DNA, 1 µl of *Rsa*I enzyme, 2 µl of 10× digestion buffer, and 15 µl of distilled water, that was incubated at 37°C for 2 hours.

2.5 Agarose gel electrophoresis

The DNA restriction fragments were separated by gel electrophoresis (0.7% agarose, TBE 0.5×), and the virus dsRNA was characterised by gel electrophoresis (0.8% agarose, TAE 1×). The images were captured with a GelDoc (BioRad) and analyzed with the Diversity Database software (BioRad).

3. Results and Discussion

The new method we propose (**Fig. 1A**) is based on that of Fried and Fink [3,4]. The two methods are the same for the earliest steps leading to cell membrane and protein structure disorganization by treating with Tris-H_2SO_4 (pH 9.3)/2% mercaptoethanol and then with SDS. This leads to the loss of selective permeability and the exit of, among others, mitDNA and dsRNA from the cell. The samples are then phenol extracted and centrifuged to obtain an enriched aqueous nucleic acid solution, mostly free of hydrophobic compounds and cell wall debris. Until this step, the handling and time consumed are the same for the two methods. The following steps are different. Cold isopropanol is used for a faster nucleic acid precipitation in the new method, instead of ethanol that is used in Fried and Fink's method. This change decreases the time for nucleic acid isolation by 30 min (from roughly 94 to 64 min). Both methods allow the isolation of the virus dsRNA as well as the mitDNA and have a similar nucleic acid yield.

The new method is also as fast (64 roughly versus 61 min) and simpler for mitDNA isolation as the already simplified method of Querol et al. [5], and no degrading enzymes are needed for cell wall digestion. This reduces the cost and also allows the isolation of dsRNA at the same time as mitDNA (**Fig. 2B** and **2B**). The Querol et al. method does not preserve RNA from degradation (**Fig. 1A**), so an alternative method is required for this purpose. Moreover, a 50 µl nucleic acid solution is obtained from 5 ml yeast culture by the new method, while only 16 µl are obtained by the Querol method, with the two similar specific mitDNA yields (**Fig. 2**).

4. Conclusions

This new method allows the concomitant isolation of yeast mitDNA and virus dsRNA, being simpler, less costly, and faster than the previous reported methods; while giving similar or greater nucleic acid yields. It facilitates the ecological study of wine yeasts by avoiding the need to use two different methods for the separate isolation of mitDNA and dsRNA, both of which can be used for yeast strain characterization.

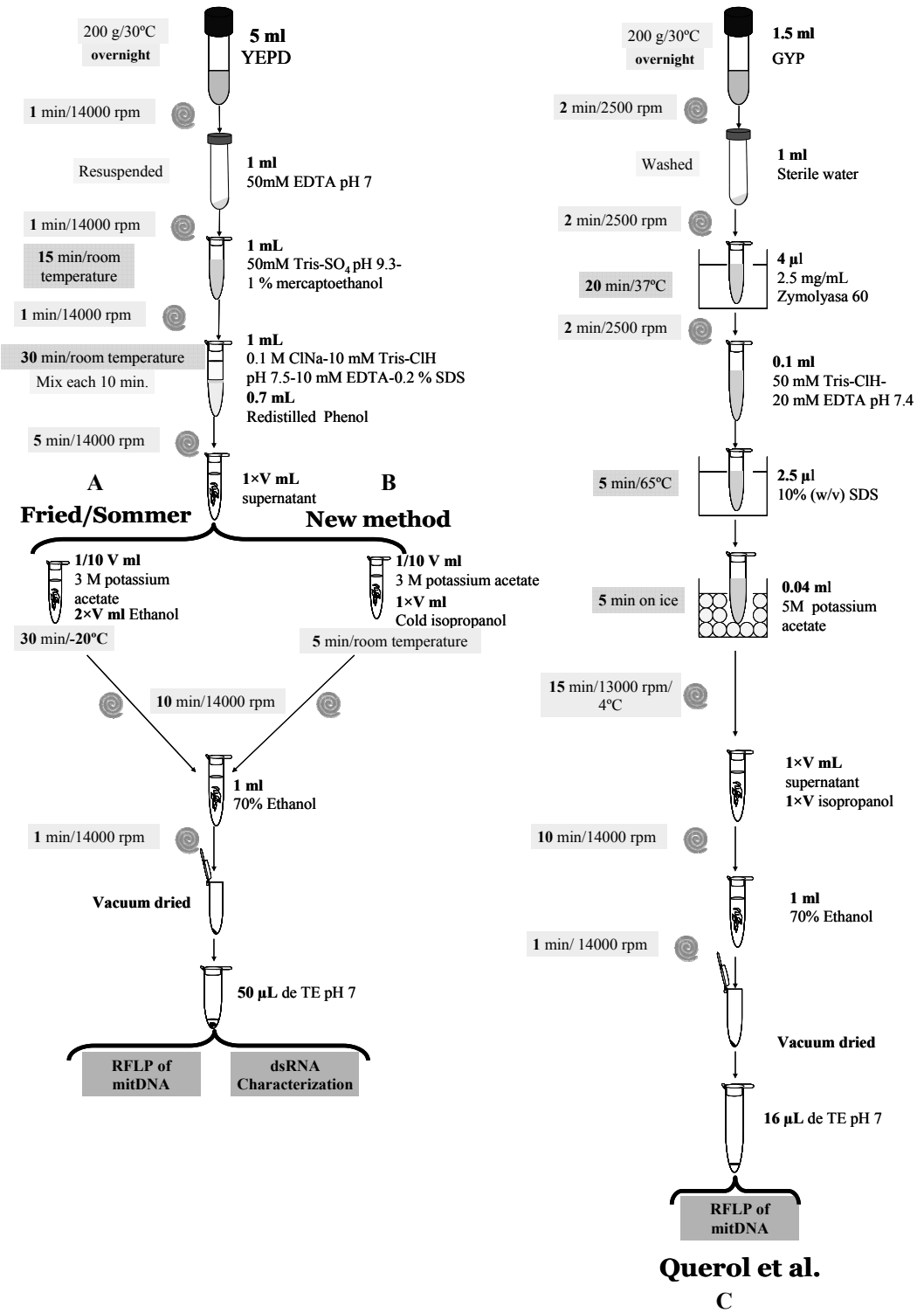

Fig. 1. Scheme to compare the three nucleic acid isolation methods. **A**: New method; **B**: Fried and Fink method (modified by Sommer and Wickner); **C**: Simplified method of Querol et al.

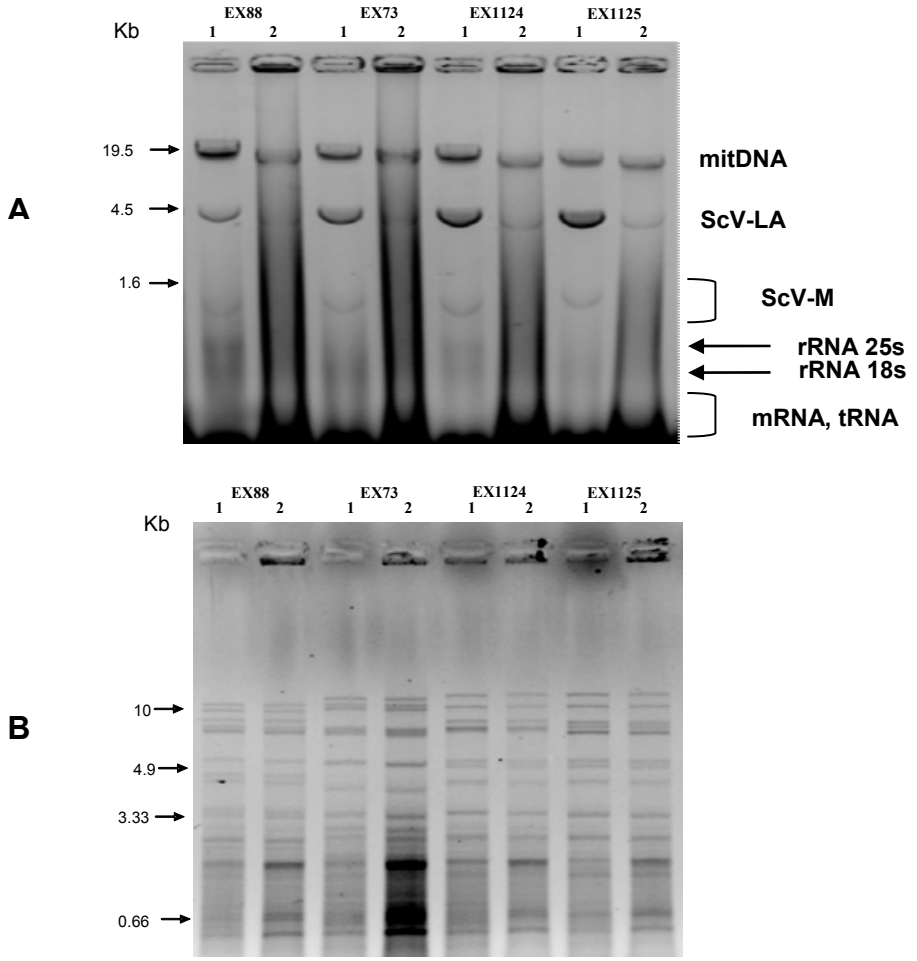

Fig. 2. Gel electrophoresis of restricted (*RsaI*) mDNA (**A**) and virus dsRNA (**B**) isolated by the new method (lines with number **1**) and the Querol et al. simplified method (lines with number **2**). **A**: Agarosa gel, 1%, TAE 1×. **B**: Agarosa gel, 0.7%, TBE 0.5×. EX88, EX73, EX1124, and EX1125 are the yeast strains analysed.

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Antioxidant substance production by the transformation of sweet potato using *Aspergillus niger*

T. Tsuruta[*,1]

[1] Laboratory of Microbial Technology, Department of Chemical Engineering on Biological Environment, Faculty of Engineering, Hachinohe Institute of Technology, 88-1, Aza-Ohbiraki, Myou, Hachinohe, Aomori, 031-8501, Japan

[*]Corresponding author: e-mail: tsuruta@hi-tech.ac.jp, Phone: +81178258036, Fax: +81178256825

The transformation of compounds liberated from sweet potato by Aspergillus niger was examined. The major product was 2-phenylethyl alcohol (PEA), followed by phenylacetic acid (PAA). Small amounts of 2-(4-hydroxyphenylethyl) alcohol (4-OH-PEA), 4-hydroxyphenylacetic acid (4-OH-PAA), 2-coumaranone, and 2-hydroxyphenyacetic acid (2-OH-PAA) were also obtained. The transformation of PEA by A. niger was next examined. PAA was not produced from PEA by this treatment indicated that the major compounds, PEA and PAA, were produced independently from sweet potato. A portion of the 4-OH-PEA was produced from PEA, and some 4-OH-PAA was produced from 4-OH-PEA. 2-Coumaranone, 2-OH-PAA, and 2, 5-dihydroxyphenylacetic acid were produced by the transformation of PAA by A. niger. 2-Coumaranone was generated by the direct oxidative cyclization of PAA by A. niger, followed by hydrolysis to give 2-OH-PAA and further oxidation to produce 2,5-OH-PAA. 2, 5-OH-PAA has high antioxidant activity.

Keywords sweet potato; pheylethyl alcohol; pheylacetic acid; 2, 5-dihydroxyphenylacetic acid; antioxidant activity; *Aspergillus niger*

1. Introduction

The Kansho-shohchuh (Japanese traditional sweet potato spirit) flavour (KSF) has been investigated in detail [1]. Several monoterpene alcohols, such as linalool, α-terpineol, citronellol, nerol and geraniol contribute to the sensory properties of KSF [1]. Additionally, these monoterpene alcohols, with the exception of citronellol, are glycosidically bound in steamed sweet potato [1, 2]. The glycosidic fraction from steamed sweet potato was hydrolyzed by almond emulsion (β-glucosidase) to release the monoterpene alcohols. 2-Phenylethyl alcohol (PEA) was also identified in KSF and produced from the hydrolysis of the glycosidic fraction of steamed sweet potato by almond emulsin[1].

The oxidation of phenylacetic acid (PAA) by microorganisms, such as *Penicillium chrysogenum,* has been investigated in relation to Penicillin production [3]. To increase the efficient usage of sweet potato for food and to establish the safety of microorganisms for food usage, we investigated the production of PEA and PAA from sweet potato by *Aspergillus niger.* Furthermore, the transformation of PAA by *A. niger* was examined, and the product and pathway were identified. The antioxidant activity of the product was also examined in this study.

2. Material and methods

2.1 Materials

2.1.1 Strains

The fungal strains used in this research was *Aspergillus niger* AHU7120, which was generously donated by the Faculty of Agriculture, Hokkaido University (AHU).

2.1.2 Sweet potato

The variety of sweet potato used in this research was Koganesengan, which is used to make kansho-shohchuh.

2.1.3 Chemicals

PAA was prepared by the hydrolysis of phenylacetonitrile by the potassium hydroxide method as described in the literature [4]. After the reaction, the reactants were acidified by concentrated hydrochloric acid and recrystallized by ethanol. 2-Coumaranone, 2-hydroxyphenylacetic acid (2-OH-PAA), 3-hydroxyphenylacetic acid (3-OH-PAA), and 2,5-dihydroxyphenylacetic acid (2,5-OH-PAA) were obtained from Acros Organics, Geel, Belgium. 2-Methyl-6-p-methoxyphenylethynylimidazopyra-dinone (MPEC) [5], which was used for the measurement of antioxidant activity, was obtained from Atto Corporation, Tokyo, Japan. Xanthine oxidase was obtained from Sigma-Aldrich, Canton, Ohio, USA. Other chemicals (guaranteed reagents) were obtained from Nacalai Tesque, Inc., Kyoto, Japan.

2.1.4 Medium

Czapek-Dox medium [6] for growing *A. niger* contained 3 g/L sodium nitrate, 0.5 g/L magnesium sulfate heptahydrates, 0.5 g/L potassium chloride, and 30 g/L glucose. After autoclaving (121 ^{o}C, 15min) the medium and allowing it to cool to room temperature, 1 mL potassium dihydrogen phosphate (containing 0.3 mg) solution, and 2 mL iron (II) sulfate heptahydrate (contained 3 mg) solution were added through a membrane filter (pore size, 0.2 μm).

2.2 Methods

2.2.1 Culture and transformation

A. niger AHU7120 was grown in 300 mL of the Czapek-Dox medium in a 500-mL flask with continuous shaking (120 rpm) for 72 h at 30 ^{o}C. Then, substrate (0.4 mmol) in 1 mL ethanol was added through a membrane filter (pore size, 0.2 μm), and shaking was continued. At the indicated time intervals, samples were removed for analysis extracted with ether, and filtered through the filter paper. The extract was dried by sodium sulfate and concentrated in vacuo.

2.2.2 Chemical analysis

The chemical structures of the products were determined by gas chromatography-mass spectrometry (GC-MS). For the GC-MS analysis, one of the products, 2-OH-PAA, was converted to 2-coumaranone by intramolecular cyclization accompanied by dehydration. Therefore, high performance liquid chromatography (HPLC) was used for quantitative analysis. All GC-MS spectra were recorded with a Hewlett Packard HP6890GC system and HP-5MS column, which was covered with 5% phenyl methyl siloxane (32m x 0.25 mm i.d.) as the liquid phase. Other conditions were as follows: carrier gas, He, flow rate 1mL/min, programming rate of column temperature, (5 min hold at 40 ^{o}C, programming rate (3 ^{o}C/min), 5 min hold at 250 ^{o}C); injection temperature, 250 ^{o}C; and HP5973 mass selective detector. HPLC spectra were recorded on a Shimadzu LC-10 system with the following conditions: pump, LC-10AD VP; detector, SPD-10A VP; column oven, CTO-10AC VP; column temperature, 40 ^{o}C; degasser, DGU-14A; flow rate 1 mL/min; detection wavelength, 254 nm; column, Shimadzu VP-ODS (150 mm x 4.6mm); and mobile phase, acetonitrile:0.2% phosphoric acid =4: 6.

2.2.3 Measurement of antioxidant activity

Ten microliters 300 μM MPEC solution, 10 μL sample, 60 μL 100 μg/mL xanthine oxidase solution, 170 μL 0.1 mM potassium dihydrogen phosphate buffer solution (pH 7.5), and 50 μL 0.72mM hypoxanthine solution were mixed, and luminescence was measured by a luminometer ATP300 (Atto Corporation).

3. Results and discussion

3.1 Transformation of sweet potato medium by *Aspergillus niger* AHU7120.

A. niger AHU7120 was cultured in sweet potato medium for 8 days. PEA was detected beginning at day 1 and increased until day 6(Fig. 1). PAA was detected from day 1 and increased until day 7. As shown in Fig. 2, small amounts of some derivatives, such as 2-(4-hydroxyphenylethyl) alcohol (4-OH-PEA), 2-coumaranone, 2-hydroxyphenylacetic acid (2-OH-PAA), and 4-hydroxyphenylacetic acid (4-OH-PAA), were also detected and production of these derivatives peaked at 5-6 days. Trace amounts of benzofran, 2-(2-hydroxyphenyl)ethyl

alcohol (2-OH-PEA), and phenylacetoaldehyde were also obtained. PEA is produced by the hydrolysis of the glycosidic fraction from sweet potato by almond emulsin (β-glucosidase) [1]. Therefore, it seems reasonable that PEA is bound as a β-glucoside in sweet potato.

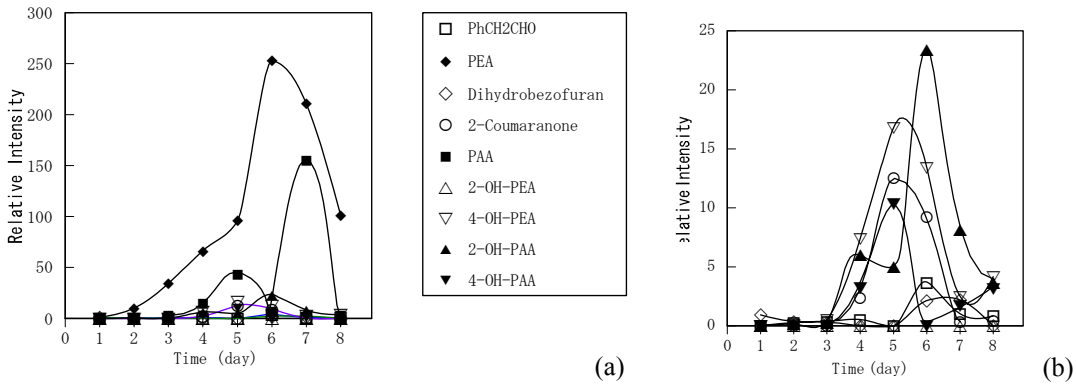

Fig. 1 Transformation of sweet potato substances by *A. niger* AHU7120. The amounts of minor products in (a) were expanded in (b).

3.2 Transformation of PEA by *A. niger* AHU7120

Next, microbial transformation of PEA by *A. niger* AHU7120 was examined. As shown in Fig. 2, small amounts of 4-OH-PEA, 4-OH-PAA, and 2-coumaranone were produced. However, PAA was not produced by the direct oxidation of PEA from sweet potato by *A. niger* AHU7120 indicating that PEA and PAA were produced independently from sweet potato by *A. niger*. PEA was shown previously to exist as a β-glucoside in sweet potato that is hydrolyzed by β-glucosidase (1). However, the production of PAA from sweet potato has not been in the literature until now.

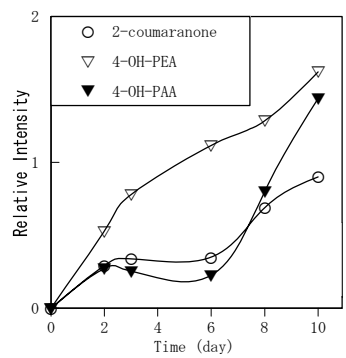

Fig. 2 Transformation of PEA with *A. niger* 7120.

3.3 Transformation of 4-OH-PEA with *A. niger*

Although PAA was not produced from PEA, a small amount of 4-OH-PAA was produced from 4-OH-PEA by *A. niger* AHU7120 (Fig. 3).

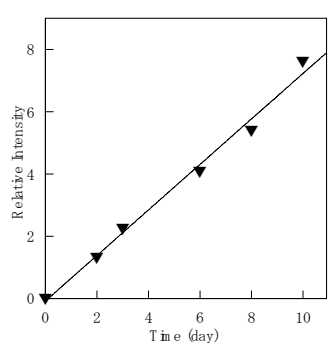

Fig. 3 Transformation of 4-OH-PEA by *A. niger* AHU7120 in Czapek-Dox medium.

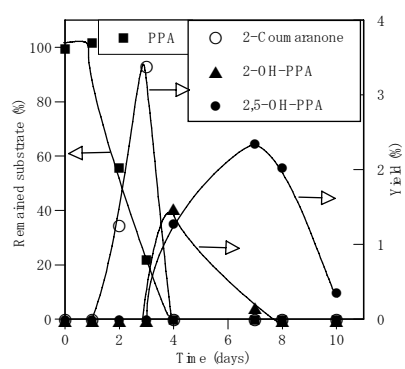

Fig. 4 Transformation of PAA by *A. niger* AHU7120 in Czapek-Dox medium.

3.4 Transformation of PAA by *A. niger*

As shown in Fig. 4, 2-coumaranone was produced 2 days after the addition of PAA to the culture of *A. niger* AHU7120. Four days after the addition, the amount of 2-coumaranone decreased sharply, and 2-OH-PAA and 2, 5-OH-PAA were produced. Therefore, it seems reasonable that 2-OH-PAA and 2, 5-OH-PAA were produced from PAA via 2-coumaranone from PAA.

3.5 Transformation of 2-coumaranone by *A. niger*

As shown in Fig. 5, 2-OH-PAA was produced 4 h after the addition of 2-coumaranone to the culture of *A.niger*, which coincided with a sharp decrease in the amount of 2-coumaranone. Eight hours after the treatment, the amount of 2-OH-PAA was decreased. 2,5-OH-PAA was produced by 47 h.

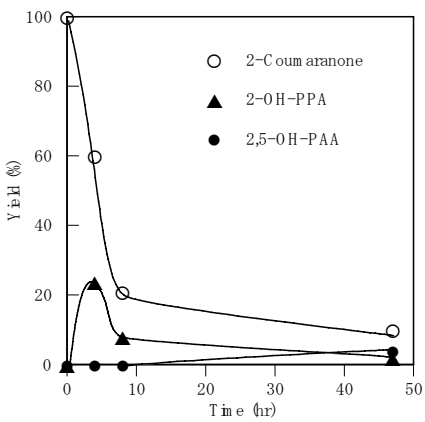

Fig. 5 Transformation of 2-coumaranone by *A. niger* AHU7120 in Czapek-Dox medium.

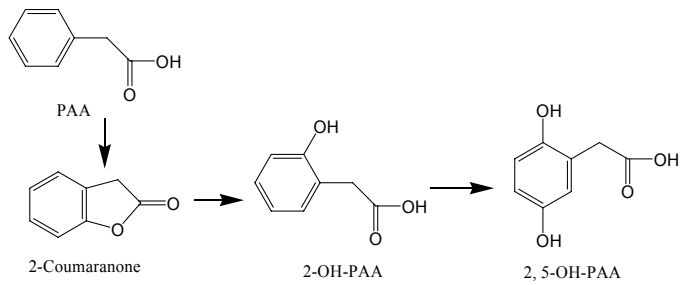

Fig. 6 Transformation pathway of PAA by *A. niger* AHU7120

These results indicate that 2,5-OH-PAA was produced from 2-OH-PAA, which was produced from PAA via 2-cumaranone (Fig. 6). 2,5-OH-PAA has been recognized as an intermediate of the metabolism of phenylalanine and PAA in some bacteria (7, 8) and in fungi (9). The absence of 2,5-OH-PAA in the metabolism of PAA by the Mulder strain of *A. niger* was reported (10), which contrasts with other studies (11, 12) that mention the production of 2,5-OH-PAA from PAA by the Mulder strain. Hitherto, the formation of PAA of 2,5-OH-PAA from PAA by other species *A. niger* has not been demonstrated. In fungi, part of the production mechanism of 2,5-OH-PAA, an established intermediate in the metabolism of PAA, has been elucidated (3). 2-OH-PAA is produced by the hydrolysis of 2-coumaranone, which in turn is produced by the oxidative intramolecular cyclization of PAA(3). 2,5-OH-PAA was produced from PAA via 2-OH-PAA (3).

3.6 Antioxidant activities of substrate, products and related compounds

2,5-OH-PAA is expected to have a high antioxidant activity due to the presence of two hydroxyl groups at the para position. Therefore, the antioxidant activities of 2,5-OH-PAA and related compounds were measured. As shown in Fig. 7, 2, 5-OH-PAA had extremely high antioxidant activity.

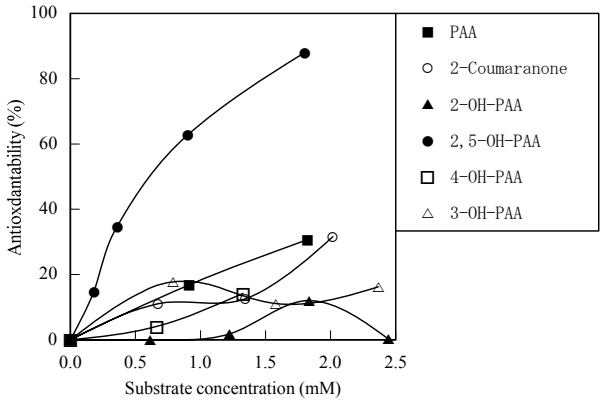

Fig. 7 Antioxidant activity of PAA and related compounds.

In summary, the present study found that 2,5-OH-PAA was generated by the microbial (*A. niger*) transformation of PAA which is produced by several plants including sweet potatoes. 2,5-OH-PAA was demonstrated to have extremely high antioxidant activity.

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Biocontrol of *Aspergillus ochraceus* by yeasts

J. Gil Serna[1], B. Patiño Álvarez[1], M.T González-Jaén[2] and C. Vázquez Estévez[1,*]

[1] Microbiology III, Fac. Biology, UCM. José Antonio Novais, 2. 28040 - Madrid. Spain
[2] Genetics, Fac. Biology, UCM. José Antonio Novais, 2. 28040 - Madrid. Spain

[*]Corresponding author : covi@bio.ucm.es. Phone: 0034913944442

Aspergillus ochraceus is one of the main contaminant of products such as coffee, grapes, cereals and derivatives. This filamentous fungus can also produce ochratoxin A (OTA), a secondary metabolite with nephrotoxic and carcinogenic properties. The maximum OTA limits allowed in food and raw agroproducts are under legal regulation. Currently, biological control has been proposed as a useful strategy in integrated management to control these fungi. Yeasts would be suitable biocontrol agents because of their characteristics: capacity of growing in fermenters, few nutritional requests and inability to produce toxic metabolites.
In this study, we tested the antagonist ability of 16 yeast strains from seven different species against five *Aspergillus ochraceus* strains. Two strains of *Debaryomyces hansenii* (CYC 1021 and CYC 1244) showed inhibitory activity against these fungi when they both were grown in YMA-MB medium supplemented with sodium chloride (6%). Additional *in vitro* assays showed that salinity enhanced biocontrol activity of *Debaryomyces hansenii* CYC 1244. The effect of temperature on biocontrol activity was also studied. The highest reduction of fungal growth was achieved at 20ºC. OTA concentration in CYA medium was significantly lower at 28ºC compared with control when fungus and yeast were co-cultured.

Keywords *Aspergillus ochraceus*; biocontrol; *Debaryomyces hansenii*; ochratoxin A.

1. Introduction

Ochratoxin A (OTA) is a secondary metabolite produced by *Aspergillus* and *Penicillium* species. This mycotoxin has been shown to have nephrotoxic, inmunotoxic, genotoxic and teratogenic properties towards several animal species [1], and has been classified by International Agency for Research on Cancer as possible carcinogen to humans (group 2B) [2]. OTA occurs in various foodstuffs and beverages including a variety of cereals, beans, groundnuts, spices, dried fruits, grapes, coffee, milk, wine and beer [3, 4] and its maximum limits on several commodities for human consumption are under legal regulation. *Aspergillus ochraceus* is an important OTA producer specie and it is considered the main source in coffee [5].

Postharvest decay can be reduced by minimizing fruit injuries, by maintaining the natural resistance of the host and by delaying senescence. However, these beneficial practices are usually not sufficient to protect the product from fungal infection [6]. The use of fungicides immediately before or at postharvest to prevent rots is being increasingly limited by legislation, because of risks for consumers´ health [7] environmental pollution, and the onset of resistant pathogen strains [8].

Biological control has been proposed as an alternative to the use of synthetic fungicides or in combination with them for reducing fungal growth and toxin biosynthesis [9, 10, 11]. Several yeast species have already been shown to be effective biological control agents in protecting plants against fungal diseases [12, 13]. Although the molecular basis of the natural process of biocontrol are still largely unknown; competition for nutrients [14], predation [15], secretion of cell wall degrading enzymes [16], killer toxins [17] or production of syringotoxins and syringomycins [18] are possible mechanisms involved in biological control.

The aim of the present work was to test the antagonistic ability of different yeast species against *A. ochraceus* strains and to determine the optimal conditions to reduce fungal growth and OTA concentration.

2. Materials and Methods

2.1. Organisms, media and culture conditions

All the isolates used in this study are given in Table 1. Yeast strains were maintained by regular subculturing on Yeast Morphology Agar (YMA) [19] of slopes at 25ºC for 48 h and subsequently stored at 4ºC until required. The *A. ochraceus* strains were maintained by regular subculturing on Potato Dextrose Agar (PDA) at 25ºC for 96 h and then stored at 4ºC until required and stored as spore suspension in 15% glycerol at –80ºC.

Table 1 Yeast and fungal strains used in biocontrol experiments.

Species	Strains			
Debaryomyces hansenii	CYC 1021	CYC 1244	CECT 10380	CECT 10386
Metschnikowia pulcherrima	L3	L4		L4.1
Pichia anomala	CECT 1114			
Pichia membranifaciens	CYC 1070			
Saccharomyces cerevisiae	CYC 1172		CYC 1174	
Torulaspora delbrueckii	CYC 1176	CYC 1177	CECT 10589	CECT 10676
Zygosaccharomyces rouxii	CYC 1150			
Aspergillus ochraceus	ALD*	ALF		AsO2*
	CECT 6795*		CECT 6825	

* OTA producers

2.2. Initial screening and salinity significance

The ability of 16 yeast strains to control *A. ochraceus* strains was tested. One ml of spore suspension (10^4 spores/ml) was cultured in YMA-MB medium either supplemented or not with sodium chloride (6%) at 20ºC. Plates were inoculated with a loopful of each yeast strain onto the surface of agar (4 yeasts/ plate). Positive biocontrol was considered when a clear zone of growth inhibition was visible after 7 days of incubation.

The effects of salinity on biocontrol of *D. hansenii* CYC 1244 against all five *A. ochraceus* strains were studied. One ml of a CYC 1244 cellular suspension (5 x 10^6 cells/ml) was mixed with 25 ml of melted YMA-MB medium with or without sodium chloride (6%). Spots of 1,5 µl of *A. ochraceus* spore suspension (10^7 spores/ml) were placed on each plate. Fungal growth was determined by measuring the fungal colony diameter at 4, 7 and 10 days. Plates were incubated at 20ºC.

2.3. Biocontrol activity: Effect of temperature and influence in OTA production

One ml of a yeast cellular suspension of *D. hansenii* (5 x 10^4 cells/ml) was mixed with 25 ml of melted CYA medium (Czapek Yeast Extract Agar). Spots of 2 µl of *A. ochraceus* spore suspension (10^6 spores/ml) were placed on each plate. Fungal growth was determined by measuring the colony diameter at 4, 7 and 10 days. This assay was carried out at 20ºC and 28ºC.

The influence of *D. hansenii* CYC 1244 in OTA concentration was analysed in the three *A. ochraceus* producers (ALD, CECT 6795 and AsO2). OTA was extracted by a method designed elsewhere [20] after 10 days of incubation in previously described conditions and measured by High Performance Liquid Chromatography (HPLC). Methanol – Monopotassium phosphate (2:1) was the mobile phase.

2.4. Statistical analysis

Statistical software SPSS 14.0 was used. Corresponding T-student test for independent or paired samples was applied. The level of significance was established as $p \leq 0.05$.

3. Results

3.1 Initial screening and salinity significance

In the initial screening we have used YMA-MB medium because it favours the growth of yeasts. Sodium chloride was added to enhance possible killer toxin production by yeasts [21]. Two out sixteen yeast strains tested in the screening, *D. hansenii* CYC 1021 and CYC 1244, showed biocontrol effect against all the *A. ochraceus* strains in assays performed in YMA-MB medium supplemented with sodium chloride (6%). Growth inhibition was not observed in experiments using YMA-MB medium without salt in these conditions. *D. hansenii* CYC 1244 was selected for additional *in vitro* assays because it produced a bigger zone of growth inhibition than CYC 1021.

Biocontrol efficiency of *D. hansenii* CYC 1244 was enhanced by high sodium chloride concentration (Table 2). Significant reduction of fungal growth (65%) by this yeast strain was observed in relation with control assays (fungi grown on free yeast plates) in YMA-MB with high salinity, while no significant reduction was observed

in experiments where the medium was not supplemented with salt. Figure 1 shows the evolution of fungal growth in YMA-MB with sodium chloride (6%).

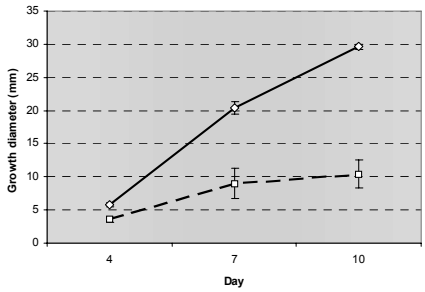

Fig. 1 Evolution of fungal growth in plates with YMA-MB supplemented with NaCl. Solid line represents control growth and discontinuous line indicates fungal growth with CYC 1244. The values are mean of five *A. ochraceus* strains. Bars indicate standard deviation.

Table 2. Effects of salinity and biocontrol activity on fungal growth. [a] Represents statistical signification ($p \leq 0.05$) when controls with or without sodium chloride were compared. [b] Represents statistical signification ($p \leq 0.001$) when control plates with fungi in YMA-MB medium were compared with YMA-MB plates where fungus and CYC 1244 were co-cultured. Values corresponded to 10-day-old cultures.

	Growth diameter without NaCl (mm)	**Growth diameter with NaCl (6%) (mm)**
Aspergillus ochraceus controls	42,40	29,60[a]
Aspergillus ochraceus + CYC 1244	36,80	10,40[b]

3.2 Biocontrol activity: Effect of temperature and influence in OTA production

This assay was carried out in CYA plates to test the effect of presence of *D. hansenii* CYC 1244 on OTA concentration. CYA is a permissive medium for OTA production [20]. The effect of temperature on both fungal growth and OTA concentration was studied at 20 and 28ºC.

The temperature showed an effect on the biocontrol efficiency against *A. ochraceus* growth (Fig. 2). Biocontrol plates incubated at 28ºC showed little reduction in growth (15.4%) compared to controls without yeast, while higher values (48.5%) were obtained at 20ºC in the same experiments.

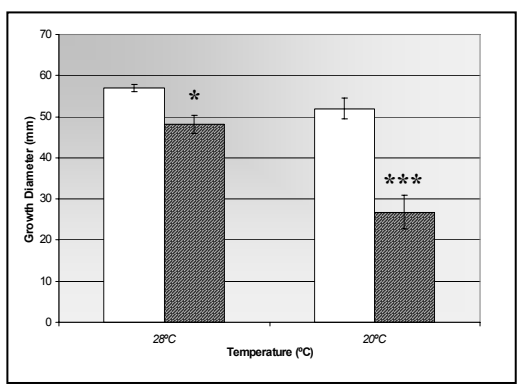

Fig. 2 Effect of temperature and presence of *D. hansenii* CYC 1244 on the reduction of fungal growth. White bars represent fungal growth in control plates without yeast and scratched bars in plates with CYC 1244. The values are mean of five *A. ochraceus* strains. Standard deviation of these values are indicated.
Statistical signification is indicated by asterisks: ***$p \leq 0.001$, **$0.001 < p \leq 0.01$, *$0.01 < p \leq 0.05$.

No OTA production by any of five *A. ochraceus* strains was detected in at 20ºC. A high reduction of OTA concentration was observed at 28ºC in plates of the three OTA-producing *A. ochraceus* strains co-cultured with *D. hansenii* CYC 1244 (Table 2).

Table 2 OTA concentration in plates at 28ºC after 10 days of incubation measured by HPLC.

A. ochraceus strain		[OTA] (μg/l)	Reduction (%)
AsO2	Control	10274	86,7
	+ CYC 1244	1371	
CECT 6795	Control	127,8	37,3
	+ CYC 1244	80,1	
ALD	Control	263	20,53
	+ CYC 1244	209	

4. Discussion

Yeasts would be suitable biocontrol agents because of their characteristics: capacity of growing in fermenters, few nutritional requests and inability to produce toxic metabolites [9]. Several yeast species seems to inhibit fungi [12, 13] and, specifically, species of *Pichia* and *Hanseniaspora* genera have been shown biocontrol against *Aspergillus ochraceus* [11]. In this work, two strains of *Debaryomyces hansenii* (CYC 1021 and CYC 1244) showed antagonistic activity against several strains of *A. ochraceus*, although the effect was more important in case of *D. hansenii* CYC 1244. This activity might be due to their ability to produce a killer toxin affecting fungal growth [17, 22]. Lethal activity of this toxin increases in mediums supplemented with sodium chloride [21]. The positive effect of high salinity found in the biocontrol assays might suggest an antagonist activity mediated by this mechanism.

Effects of temperature on growth and OTA production by *A. ochraceus* have been reported [23, 24]. In this work, we have observed antagonist activity at both temperatures tested, 28 ºC and at 20ºC. However, reduction of fungal growth was more drastic at 20ºC than at 28 ºC in all strains studied. This fact also supports the theory of a killer toxin mediated mechanism of biocontrol by *D. hansenii* CYC 1244 since it has been reported that toxin stability decreases at temperatures higher than 20ºC [17]. Additional experiments will be performed to rule out other possibilities such as competition.

The antagonist activity had also a remarkable effect on OTA reduction when *D. hansenii* CYC 1244 and *A. ochraceus* were co-cultured in the same plate, with values reaching 86% in AsO2 strain. In some studies, reduction of OTA concentration by yeast species might be achieved by the ability to adsorb or to retain mycotoxins [25]. Moreover, it has been described the capacity of several yeast species to produce volatile compounds that affect OTA production by *A. ochraceus* [26]. Both mechanisms could be possible in our case, although we do not have information yet about the mechanism underlying the effect of *D. hansenii* in OTA reduction.

The results obtained in the present work indicate a positive antagonistic effect by *D. hansenii* on all the *Aspergillus ochraceus* strains showed by reduction of fungal growth and OTA concentration in *in vitro* cultures. Further studies *in vivo* are needed to check if it is possible to apply this yeast in biological control of this fungus.

Acknowledgements: This work was supported by the Spanish MEC (AGL 2004-07549-C05-05/ALI) and UCM-CM (CCG06-UCM/AGR-1281). J. Gil-Serna was supported by a FPU fellowship from the Spanish Ministery of Education and Science.

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Characterization of killer wine yeasts from spontaneous must fermentation in six wine producing zones of southwestern Spain

M. Maqueda Gil[1], M. L. Álvarez[2], E. Zamora[2] and M. Ramírez Fernández[1,*]

[1] Departamento de Ciencias Biomédicas Área de Microbiología, Edificio Juan Remón Camacho, Avda. de Elvas s/n. 06071 Badajoz, Spain.
[2] Estación Enológica, Conserjería Agricultura y Medio Ambiente. Junta de Extremadura. Almendralejo, Badajoz, Spain.

[*]Corresponding author: e-mail: mramirez@unex.es, Phone: +34 924289426, Fax: +34 924289427

Some yeast strains secrete protein toxins, encoded in dsRNA virus (ScV-M), that are lethal to sensitive strains of different species and genera, and have been designated as killer yeasts. We analyzed 1114 wild *Saccharomyces* yeasts from spontaneous must fermentations in six wine producing zones. Among them, 61.2% were non-killer, and 38.8% killer. Four killer phenotypes were found. The most frequent (28.2%) belonged to the previously reported K2 type encoded by ScV-M2 (dsRNA size of 1.1-1.75 kb). Two newly found phenotypes are encoded in the dsRNA of two new ScV-M particles: K*lus* (7.1%, dsRNA size of 2-2.5 kb); and K*anas* (1.5%, dsRNA size of 1-1.75 kb). The fourth, K*pac* (2%) is not encoded in any known ScV-dsRNA type. Any of this type of killer yeast is able to kill any of the other three types. While K2 yeasts were found in all the geographic zones and vintages studied, K*lus* and K*anas* were only found in the warmest zones during the hottest and driest vintages.

Keywords wine yeast; killer phenotype; dsRNA virus; spontaneous fermentation.

1. Introduction

The fermentation of grape juice is a complex microbiological process involving interactions between yeasts, bacteria, fungi, and their viruses, with the yeasts playing the most important role. Some yeast strains secrete protein or glycoprotein toxins, encoded in dsRNA virus (ScV-M) of the *Totiviridae* family, that are lethal to sensitive strains of different species and genera, and have been designated as killer yeasts. So far, in *S. cerevisiae* three major killer viruses have been discovered (ScV-M1, ScV-M2, and ScV-M28), which encode a specific killer toxin (K1, K2, and K28, respectively), and a self-protective immunity component [1-3]. In each case, the killer phenotype requires the presence of two different dsRNA viruses: an LA helper virus and the toxin-encoding (M) killer virus. *In vivo*, both dsRNA genomes are separately encapsidated into virus particles that stably persist in the cytoplasm of the infected yeast cell. The ScV-LA particle alone does not confer a phenotype upon its host nor does it lead to cell lysis or slow cell growth. All known fungal viruses spread horizontally by cell–cell mating or heterokaryon formation. The killer activity could be a mechanism of competition, because these yeasts when present can come to dominate must fermentation. Recently, Gulbiniene et al. [4] found a new ScV-M particle in a *S. cerevisiae* strain isolated from apple juice. These particles were similar to ScV-M28 but their killer activities and immune properties were different from the K28 phenotype. Although the killer phenotype has been described in several yeast species, the highest frequency has been found among strains of *S. cerevisiae*.

2. Materials and Methods

2.1. Yeast strains

The 1114 wild indigenous yeasts were isolated from spontaneous must fermentations in six wine producing zones of Extremadura (SW Spain): Tierra de Barros, Ribera Alta, Ribera Baja, Matanegra, Cañamero, and North of Cáceres.

2.2. Plate assay for killer activity at different pHs

Production of killer toxin by yeasts was analysed in MB medium at different pHs (4, 4.7, and 6) and temperatures (20, 28 and 30ºC). Plates were seeded with 48-hour cultures of *Saccharomyces cerevisiae* strains with different phenotypes (**Table 1**). Strains to be tested for killer activity were loaded onto the seeded agar to produce patches of about 4-5 mm diameter. The killer yeasts produced growth-free or dead zones around themselves (**Figure 1**).

Table 1. *S. cerevisiae* strains used to analyze killer phenotype

Strain	Genotype	Origin
EX33	*MATa/α HO/HO* [k1^0; k2^0; k28^0; k*lus*^{0}]	J. A. Regodón[a]. D.O. Ribera del Guadiana
F166	*MATα leu1 kar1 LA-HNB M1* [k1$^+$]	J.C. Ribas[b] (from R.B. Wickner)
EX73	*MATa/α HO/HO LA M2* [k2$^+$]	J. A. Regodón. D.O. Ribera del Guadiana
F182	*his2 ade/leu2-2 ura3-52 ski2-2 L-A M28* [k28$^+$]	J. C. Ribas (from M. Schmitt)
EX198	*MATa/α HO/HO LA Mlus* [klus$^+$]	M. Maqueda[c]. D.O. Ribera del Guadiana

[a] J. A. Regodón, Departamento de Química Analítica, Universidad de Extremadura, Badajoz, Spain.
[b] J. C. Ribas, Departamento de Microbiología y Genética, Universidad de Salamanca, Spain.
[c] M. Maqueda, Departamento de Ciencias Biomédicas, Área de Microbiología, Universidad de Extremadura, Badajoz, Spain.

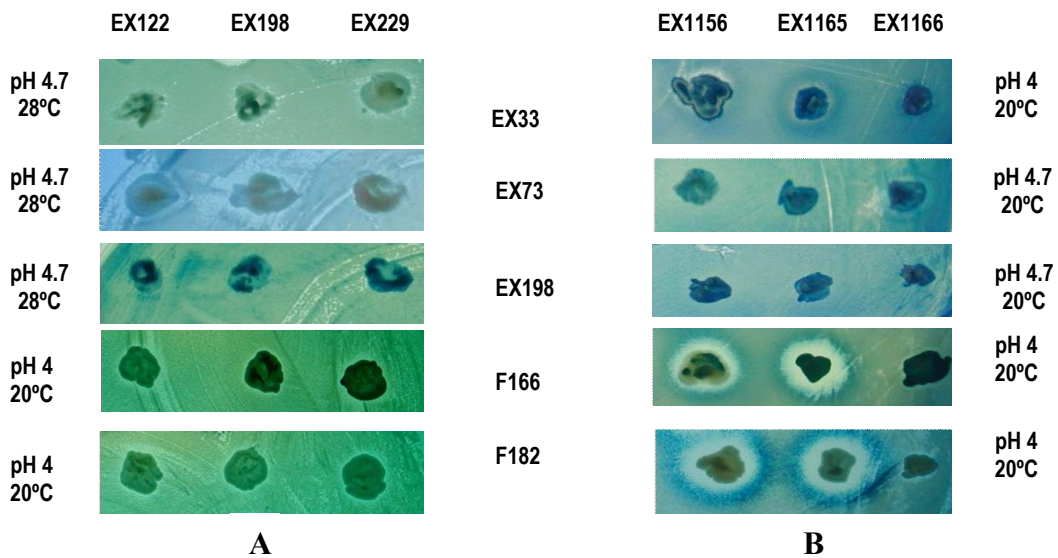

Fig. 1. Killer plate assay for K*lus* (**A**) and K*pac* (**B**) yeasts. Each row of images belongs to tests seeded with the strain given in the central column: **EX33** (K$^-$, sensitive); **EX73** (K2); **F166** (K1); **F182** (K28); and **EX198** (K*lus*). The temperature and pH are given at each side of the images. The killer wild wine yeasts EX122, EX198, and EX229 are K*lus*; and EX1156, EX1165, and EX1166 are K*pac* type.

2.3. Isolation and characterization of ScV-M particles

The dsRNA of the viruses were isolated by the method of Fried and Fink [5] modified by Sommer [6], and characterized by gel electrophoresis (0.8% agarose, TAE 1×). The dsRNA size was determined by comparing with molecular weight markers: 1kb (*GIBCOLAB*), 1 kb (*Biorad*), and 500 bp (*Biorad*). The images were captured with a GelDoc (BioRad) and analyzed with the Diversity Database software (BioRad).

3. Results and Discussion

3.1. Types of killer yeasts from SW Spain

The presence of 26.7% K2 killer *Saccharomyces cerevisiae* yeasts in the SW-Spain vineyard-winery ecosystem has been described previously [7]. We analyzed the killer phenotype of 1114 newly isolated wine *Saccharomyces* yeasts. 61.2% were non-killer (K$^-$) and 38.8% were killer (**Table 2**, **Figure 1**), a higher proportion than previously reported [7]. Most killer yeasts belonged to the K2 type, 28.2% of the total population. Additionally, three novel types of killer yeasts were found: K*lus*, K*anas*, and K*pac*.

Table 2. Percentage of each killer type among the 1114 wild isolated yeasts, and proportion of yeast bearing each ScV particle among each killer yeast type.

Phenotype	%	ScV-LA		ScV-M				
		-[a]	+	-[a]	M2	M*lus*	Other[b]	M*anas*
K-	61.2	66.4	32.9	76.7	16	2.5	4.8	0
K2	28.2	0	100	0	100	0	0	0
K*lus*	7.1	0	100	0	0	100	0	0
K*pac*	2	100	0	86.4	0	0	13.6	0
K*anas*	1.5	0	100	0	0	0	0	100

[a] Absence of ScV particle

[b] Presence of undetermined nucleic acid other than ScV-M dsRNA.

The K*lus* yeasts, 7.1 %, killed the sensitive yeast and the K1, K2, and K28 killer yeasts (**Fig. 1A**). They were weak killer (growth inhibition halo less than 1 mm thick), and their optimal killing condition varied depending on the lawn seeded yeast, pH, and temperature. In particular, they showed highest killer activity against other indigenous wine yeasts, EX33 (sensitive) and EX73 (K2), at pH 4.7 and 28ºC; while they showed the highest killing activity against the laboratory haploid strains F166 (K1) and F182 (K28) at pH 4 and 20 ºC (**Fig. 1A**).
The killer phenotype of K*pac* yeasts, 2%, was mainly dependent on the lawn seeded yeast. Most K*pac* yeasts were weak killer, and just against the non-killer yeast EX33. Only 13.3% of K*pac* yeasts killed other killer yeast type (**Fig. 1B**), mostly K1 and K28. The only K*pac* strain able to kill K2 yeasts was EX1166 (**Fig. 1B**). No K*pac* strain killed any K*lus* yeast.

K*anas* yeasts, 1.5%, were killer to most of the other killer yeast types, even to K*lus* in some instances (**Fig. 2**). They can be grouped into two categories: that of EX235 and EX805 (**Fig. 2**) that clearly killed EX73 (K2) strain in different conditions, and also killed EX33 only at pH 6 and 30°C (**Fig. 2B**). These yeasts barely killed any other yeast at the lowest pH and temperature tested, pH 4 and 20°C (**Fig. 2A**). The second category of K*anas* yeasts, that of EX234 and EX1063, killed K^-, K1, K2, and K28 strains at the lowest pH and temperature tested (20°C and pH 4 –K^-, K1, and K28– or 4.7 –K2– **Fig. 2A**). The 16.7% of K*anas* yeasts killed the K*lus* strains, but only at the highest pH and temperature tested, pH 6 and 30°C (**Fig. 2B**).

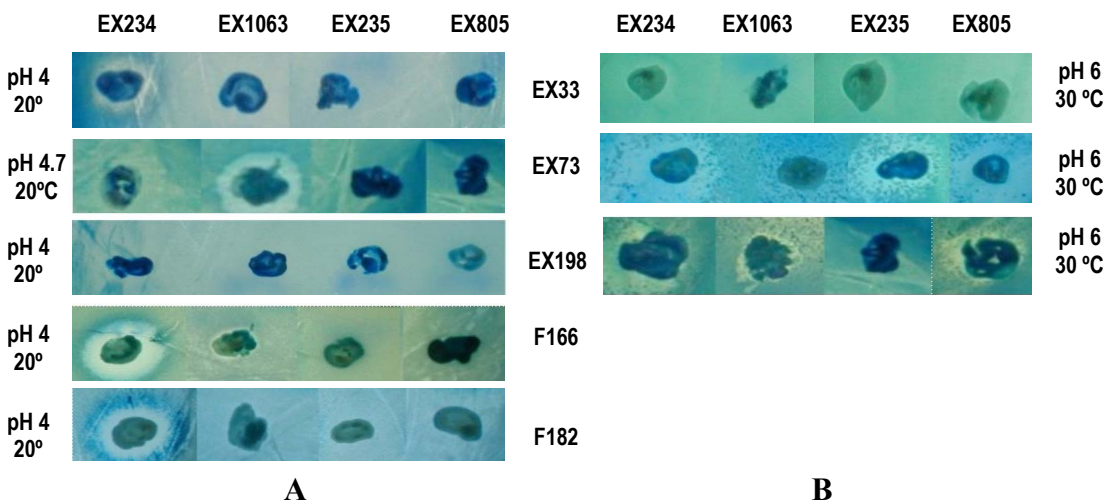

Fig. 2. Killer phenotype test of K*anas* yeasts at different pH and temperature (**A** & **B**). Each row of images belongs to tests seeded with the strain given in the central column: **EX33** (K^-, sensitive); **EX73** (K2); **F166** (K1); **F182** (K28); and **EX198** (K*lus*). The temperature and pH are given at each side of the images. The killer wild wine yeasts EX234, EX1063, EX235, and EX805 are K*anas* type.

The most frequent killer yeasts, K2, were found in all the different vineyard zones studied, and were easily isolated from most spontaneous must fermentations of all the vintage years. However, K*lus* and K*anas* yeasts were only found in four zones: Tierra de Barros, Matanegra, Ribera Alta, and Ribera Baja. These are the warmest zones analyzed, they are very close to each other, and there is much grape trading between the local wineries, which may facilitate K*lus* and K*anas* yeast dispersion. Also, these killer yeasts were isolated more frequently during the driest vintages.

3.2 Characterization of the dsRNA from the new ScV-M particles

There was no viral particle in 45.5% of the yeasts analyzed. However, the ScV-LA particle was found in 57.5% of them (in 96.6% of the killer yeasts, and 32.9% of the non-killer).

Of the killer yeasts, 98.8% also contained one type of ScV-M particle; in the other 1.2%, no ScV-M particle was detected, raising the possibility of a nuclear genome encoding for the new killer phenotype. This situation only occurred for the K*pac* yeasts (**Fig. 3**), and it could be similar to what is the case with killer toxin *KHR* and *KHS* studied by Goto et al. [8,9].

All K2, K*lus*, and K*anas* yeasts had ScV-LA plus one type of ScV-M (**Fig. 3**). The K2 and K*anas* yeasts had the lowest molecular weight dsRNA, 1.1-1.75 kb. The dsRNA of the viruses present in the K*lus* yeasts (ScV-M*lus*) had the highest molecular weigh so far reported for *S. cerevisiae* ScV-M particles (2-2.5 kb, **Fig. 3**). We confirmed that it really is dsRNA (it was degraded by RNAse A, and not degraded by DNAse, data not shown), and that it encoded for the K*lus* phenotype (the killer phenotype disappeared after curing the ScV-M*lus* with 2µg/µl of cycloheximide).

Finally, we would indicate that 18.5% of the non-killer yeasts bore ScV-LA plus a ScV-M particle (M2, 16%; or M*lus*, 2.5%). For some reason (genetic or lack of the proper test condition), these yeasts did not show the killer phenotype.

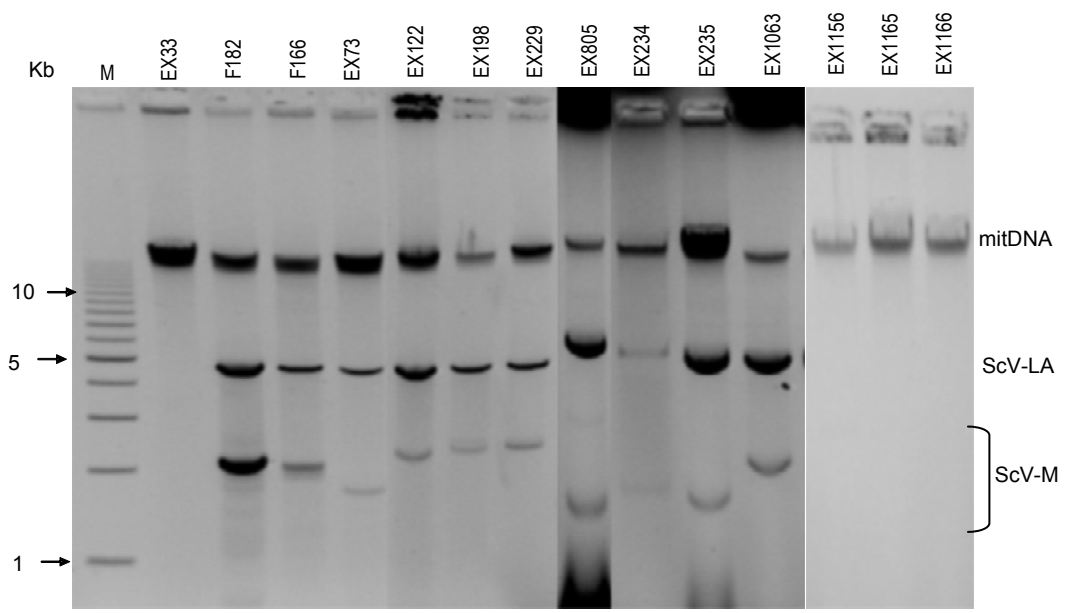

Fig. 3. Characterization of dsRNA of ScV-M particles or killer yeasts (electrophoresis in 0.8% agarosa gel, TAE 1×). Each line belongs to a different yeast. **M**: Molecular weight markers 1Kb (Biorad). EX33 (sensitive), EX73 (K2), F166 (K1), and F182 (K28) are killer yeast type described previously. EX122, EX198, and EX229 are K*lus* yeast*s*. EX805, EX234, EX235, and EX1063 are K*anas*. EX1156, EX1165, and EX1166 are K*pac*.

4. Conclusions

Among the analyzed 1114 wild *Saccharomyces* yeasts from spontaneous must fermentations, 61.2% were non-killer, and 38.8% killer. Four killer phenotypes were found. The most frequent (28.2%) belonged to the previously reported K2 type encoded by ScV-M2 (dsRNA size of 1.1-1.75 kb). Two newly found phenotypes are encoded in the dsRNA of two new ScV-M particles: K*lus* (7.1%, dsRNA size of 2-2.5 kb), and K*anas* (1.5%, dsRNA size of 1-1.75 kb). The fourth, K*pac* (2%), is not encoded in any known ScV-dsRNA type. Any of this type of killer yeast is able to kill any of the other three types. While K2 yeasts were found in all the geographic zones and vintages studied, K*lus* and K*anas* were only found in the warmest zones during the hottest and driest vintages.

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Conjugated linoleic acid: a multifunctional nutraceutical from the rumen

A. K. Puniya*, S. Chetanya, A. K. Tyagi[1], R. Nagpal, S. Kumar, M. Malik, M. Puniya and K. Singh

Dairy Microbiology Division, [1]Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal 132001 (Haryana), India

*Corresponding author: Tel: +91 184 3292844 (Res); +91 184 2259176 (Off); +91 9354116204 (Cell); +91 184 2250042 (Fax); E-mail: puniya1234@rediffmail.com

Milk fat generally had a bad image for its relatively high content of saturated fatty acids that may increase plasma cholesterol in consumers. However, in the recent past, a lot of attention has been directed toward conjugated linoleic acid (CLA) that is naturally present in milk and its products. The formation of CLA is a part of process called biohydrogenation that takes place in the rumen, which converts linoleic acid to stearic acid. Hence, ruminants are the major reservoir for this fatty acid. Though, the presence of CLA in milk was known for a long time, there has been an explosion of interest in CLA research in the last two decades after the discovery that it possesses potential anticarcinogenic, antiatherogenic, anticholestrolemic and immuno-modulatory health benefits.

Keywords Conjugated linoleic acid; CLA; milk fat; rumen; fatty acids.

1. Introduction

Fatty acid research has amplified since the comprehension that there are a number of fatty acids, generally conjugated fatty acids and primarily conjugated linoleic acid (CLA), have potential health implications in human health. A number of health benefiting properties associated with the isomers of CLA has attracted the researchers. It is unique because unlike most naturally occurring anticarcinogenic substances found mainly in plants, it is present in food from animal sources.

CLA is naturally present in dairy products, meats of different origins and human plasma phospholipids. It is also found in animal tissues and other food sources i.e. poultry and eggs that have undergone heat treatments. Though, vegetable fats are poor sources of CLA, yet, it is produced from linoleic acid in safflower and sunflower oil after special treatment of these oils. There are evidences that human milk contains CLA. However, ruminant milk is the richest dietary source of CLA, mainly the cis-9, trans-11 isomer [1]. The cis-9, trans-11 isomer of CLA, also known as rumenic acid, is produced in the rumen via microbial metabolism of linoleic (LA) and linolenic (LNA) acids. Another CLA isomer of ruminant tissues is trans-10, cis-12 CLA. CLA has been widely recognized as a supplement for human nutrition and has shown several health benefits on consumption.

The recent attention focused on CLA can be explained by the plethora of potential health benefits attributed to this unique fatty acid. For example, CLA is a potential promoter of leanness; hence, cardiovascular health of consumer may be improved by lowering the risk of diet-based disorders. It also serves as a protection from catabolic effects of immune stimulation and its supplementation shows various responses on lipid profiles. CLA is also reported to have anti-diabetic effects by improving insulin sensitivity [2]. Most of the commercial CLA preparations mainly contain cis-9, trans-11 CLA and trans-10, cis-12 CLA along with smaller amounts of other isomers.

2. Biosynthesis of CLA by rumen bacteria

CLA is produced in ruminant animals as an intermediate in the biohydrogenation of dietary linoleic acid by a linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* [3, 4]. It is also formed in the mammary gland by the endogenous conversion of t-11 C18:1 (transvaccenic acid, TVA), another intermediate of rumen biohydrogenation of LA or linolenic acid, by the desaturase enzyme [5]. LA biohydrogenation starts with isomerization, where double bond at carbon-12 position is transferred to carbon-11 forming c-9, t-11 CLA, followed by rapid hydrogenation of cis-9 bond leaving behind TVA. The enzyme for conjugation of cis-9, cis-12 double bonds is linoleic acid isomerase bound to bacterial cell membrane [6].

With a low fiber diet, a change occurred in the transoctadecenoic acid profile of milk and being trans-10 isomer predominant in fat [7]. This led to propose another pathway for the ruminal synthesis of t-10, c-12 CLA

involving bacterial c-9, t-10 isomerase with the formation of a t-10, c-12 double bond as the first step [6]. The c-12, t-11 isomerase from *B. fibrisolvens* hydrogenate t-10, c-12 octadecadienoic acid thus producing t-10 octadecenoic acid. Nearly half of the LA is converted to t-10, c-12 isomer of CLA and only 10% is converted to t-10 octadecenoic acid by *Propionibacterium* [8].

Megasphaera elsdenii have also been found to produce t-10, c-12 isomer of CLA in the rumen [9]. The t-10, c-12 isomer was formed from LA but not from either of the LNA as was the case with c-9, t-11 isomer of CLA. Mosley et al, [10] have shown that oleic acid also forms various trans C18:1 including TVA during its biohydrogenation to stearic acid. Oils and seeds of peanuts, rapeseed, palm, canola, sunflower etc. contain higher proportion of oleic acid that may enhance the milk fat CLA from ruminants when fed.

Mainly two types of bacteria group A and group B are involved in biohydrogention process [11]. Group A bacteria hydrogenate linoleic and linolenic acid to trans-11 octadecadienoic acid, and are not able to or incapable of hydrogenating octadecadienoic acid. Examples are Butyrivibrio, Micrococcus, Ruminococcus, and Lactobacillus. Group B bacteria are capable of hydrogenating a wide range of octadecadienoic acids, including cis-9 (oleic) and trans-11 (TVA) acids as well as linoleic acid to stearic acid. Examples are *Fusocillus* spp and gram negative rods.

Jiang et al, [12] reported the formation of CLA from linoleic acid by *Propionibacterium freudenreichii*. Lin et al, [13] and Ogawa et al, [14, 15] reported the production of CLA from free linoleic acid by *Lactobacillus acidophilus*. Kishino et al, [16] found that *Lactobacillus plantarum* formed high levels of CLA from free linoleic acid upon extended incubation. Bifidobacteria also produce CLA, mainly the *cis*-9, *trans*-11 isomer [17]. Several strains of probiotic lactobacilli and bifidobacteria are capable of converting linoleic acid to CLA [15, 18, 19]. *Lactobacillus brevis* has been found to produce significantly higher amount of CLA (10.53 mg CLA/ g fat) using sunflower oil (0.25%) as a substrate, when compared to control [20].

3. Biological activities of CLA

3.1. Anticarcinogenic activity and Immune functions

CLA has been repeatedly shown to have anticarcinogenic effects in animal models for stomach neoplasia [21], mammary tumors [22], and skin papillomas [23]. CLA is effective in reducing the size and metastasis of transplanted human breast cancer cells and prostate cancer cells in severely compromised immunodeficient mice [24]. This property of CLA is in sharp contrast to the pro-cancer activity associated with feeding linoleic acid [25].

A study suggested that CLA might act by antioxidant mechanisms [21], others suggested inhibition of nucleotide synthesis [26] or inhibiting both DNA-adduct formation [27] and carcinogen activation, as opposed to direct interaction with the pro-carcinogen, scavenging of electrophiles or selective phase I detoxification pathways [28]. Cook and Pariza, [29] hypothesized that CLA indirectly affects the function of tumor necrosis factor-α and interleukin-1.

3.2. Fat reducing activity

Dietary CLA has been shown to increase the level of total saturated fatty acids whereas monounsaturated fatty acids are decreased due to regulation of stearoyl-CoA desaturase activity by t-10, c- 12 CLA isomer [30]. Studies conducted in dairy cows demonstrated that abomasal infusion of CLA decreased milk fat yield. Those effects may be due to CLA isomers containing a t-10 double bond [31]. In a study conducted in obese humans, CLA was shown to reduce body fat mass and that no additional effect on body fat mass was achieved with doses >3.4 g CLA/d [32].

3.3. Antiatherogenic activity of CLA

There are few studies indicating that CLA may also reduce the risk of cardiovascular diseases in animal models [33]. A study conducted in rabbits showed that dietary CLA resulted in a marked decline in the levels of total plasma cholesterol, triacylglycerol, and the ratio of low-lipid to high-lipid cholesterol [34], but not in the c-9, t-11 CLA group [35].

Munday et al, [36] reported that CLA actually did not reduce the incidence of atherosclerosis, but increased the incidence of fatty acid streaks in mice compared to control-fed mice. The mice fed CLA had significantly lower concentration of serum triacylglycerol and a significantly higher ratio of serum HDL-cholesterol: total cholesterol.

3.4. CLA and diabetes

Recently, a significant production of CLA was observed in the fermented milk containing *L. acidophilus* and *L. casei* that had an anti-diabetic effect in mice [19, 37]. Nonetheless, if CLA can improve glucose homeostasis and inhibit body fat accretion as demonstrated in mice, rats, and pigs, then CLA may be beneficial to humans prone to diabetes.

3.5. Effect of CLA on energy intake

Rats, mice and chickens fed CLA enhanced diets tended to consume less feed for equivalent or greater weight gain [38]. The reduced intakes of food associated with the intake of CLA are not considered to be significant enough to account for the observed reduction in fat deposition [39]. Along with reducing energy intake, CLA has been shown to increase energy expenditures by animals through increased metabolic rate [40]. Increased energy expenditure has been linked to CLA's effects on uncoupling protein 2, a major uncoupling protein of white adipose tissue.

4. Dietary approaches in producing milk with improved quantity of CLA

A diversity of factors, such as the cow's diet, can affect the CLA content of milk fat. Because the CLA content of dairy products is related to their fat content, CLA levels are greater in higher fat than in lower fat products. The finding that various dietary manipulations can increase the CLA content of milk fat may open the door for CLA-enriched dairy foods. Consequently, dietary modifications have been used to increase the natural production of CLA in bovine milk fat.

A study showed that the concentration of CLA in the milk could be enhanced by the addition of sunflower oil and linseed oil (high in linolenic acid) in ruminant diets [41]. Another study conducted in goat showed that canola oil in the diet increased the levels of CLA in the milk compared to control [42]. Dhiman, et al, [43] reported a higher milk CLA concentration with pasture feeding compared with total mixed rations (TMR) feeding. Similar enrichment of CLA has often been achieved when the TMR was supplemented with unsaturated fat from oilseeds peanut oil, sunflower oil or linseed oil [41].

The increase in CLA levels observed with the sunflower oil treatment represented levels significantly greater than those typically seen in traditional diets. The level of CLA obtained using supplemental fat varies to a large extent depending on the ruminal conditions. Bell and Kennelly, [44] carried out a feeding trial to manipulate the animal's diet in a way that would increase the CLA content. The study showed that milk fat can be modified to give a more favorable composition. Furthermore, it demonstrated the feasibility of producing CLA enriched milk using modifications to the diet of the cow.

5. Conclusion

Most research on CLA to date is in *in-vitro*, and animal/ human trials have been initiated recently. Yet, much remains to be known about the exact mechanisms by which CLA exerts its diverse physiological effects. New evidences are emerging that CLA in dairy foods may be beneficial to health including tumor control, promotion of metabolic function, lowering of body fat, and lessening of arteriosclerosis. Yet, how CLA accomplishes all these functions in the living system is still not fully known, and is a matter of continuous research. Hence, CLA can result in increased demand for consumption of dairy foods as well as nutraceutical based functional foods, provided its potential benefits are well authenticated further.

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Effect of the baking process on the reduction of ochratoxin A in wheat flour

F.M. Valle-Algarra[1], R. Mateo[1], Á. Medina[2], F. Mateo[3], E.M. Mateo[2], J.V. Gimeno-Adelantado[1], and **M. Jiménez[*,2]**

[1] Dpto. de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjassot, Valencia, Spain
[2] Dpto. de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjassot, Valencia, Spain
[3] Dpto. de Ingeniería Electrónica, Universidad Politécnica de Valencia, Camino de Vera 14, E-46022, Valencia, Spain

[*]Corresponding author: e-mail: misericordia.jimenez@uv.es, Phone: +34 963543144

An effective treatment for eliminating ochratoxin A (OTA) from food products is essential to minimize human exposure to this toxin. Many physical and chemical methods such as cleaning, milling, or baking have been proposed to reduce mycotoxin contamination of wheat products. In this study, the influence of the baking process on the stability of OTA in wheat flour spiked at different toxin levels has been evaluated. The results showed that fermentation and oven-heating have significant effects on the toxin removal. After the baking process, the reduction level of toxin ranged between 67.1% and 75.5% in the wheat flour spiked with 2 ng of OTA/g. In addition, OTA stability inside the bread and on its external surface was studied. The obtained results indicated that OTA reduction rate inside the bread is significantly lower than in the outer part.

Keywords fermented dough; wheat flour; ochratoxin removal; baking process

1. Introduction

Cereals are susceptible to be contaminated with mycotoxins and the fungi able to produce them. Usually these fungi are species of the genera *Fusarium, Aspergillus* and *Penicillium* [1-3]. It has been demonstrated that different products of massive consumption in Europe and other countries like bakery products are frequently contaminated by ochratoxin A (OTA). [4]. OTA has been found to be a potent renal toxin in all the animal species tested. It induces a typical karyomegaly and a progressive nephropathy [5].

Thus, an effective treatment for eliminating OTA from cereal products is essential to minimize human exposure to the toxin. The results from the literature suggest that during food processing fractionation or reduction occurs [6]. Many physical and chemical methods have been used to reduce mycotoxin contamination of foodstuffs and feedstuffs, such as segregation of contaminated from non-contaminated kernels in water and saturated sodium chloride, milling, cleaning or washing, sieving and dehulling [7]. In terms of cooking treatment methods, some reports showed that only a small further reduction occurred when the dough was baked into bread or cookies. OTA is relatively stable once formed, but by effect of high temperature, acidic or alkaline conditions or in the presence of enzymes some breakdown can occur [8-11].

The aim of this study was to evaluate the influence of the baking process on the OTA content in bread baked with wheat flour spiked with this mycotoxin at different levels to establish how much this process reduce the contamination level.

2. Experimental

2.1 Chemicals and reagents

OTA standard was supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile, methanol and acetic acid were purchased from J.T. Baker (Deventer, Holland). All solvents were LC grade. Filter papers (Whatman No 4) was from Whatman (Maidstone, UK). Immunoaffinity clean-up column OchraTest™ was made by Vicam (Vicam Science Technology, MA, USA). Phosphate-buffered saline solution (PBS, pH 7.4) was prepared with 0.2 g of potassium chloride (Panreac, Montcada i Reixac, Barcelona, Spain), 0.2 g of potassium dihydrogen phosphate (Sigma), 1.16 g of anhydrous disodium hydrogen phosphate (Panreac) and 8.0 g of sodium chloride (J.T. Baker) in 1 l of pure water. Pure water was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required.

Compressed yeast cake was purchased from supermarket, and stored at 4 ºC. Wheat flour used to prepare the dough was contaminated with 2, 5, and 10 µg of OTA/kg.

A stock solution of OTA was prepared by solving 1 mg of OTA in 2 ml of toluene–acetic acid (99:1, v/v). A series of working standards was prepared by evaporation of known aliquots of the stock solution and dissolution in filtered LC mobile phase.

2.2 Dough preparation and bread making

Dough was made with wheat flour, compressed yeast cake, water and of sodium chloride. (50:2:30:1, w/w/w/w). Dry ingredients were mixed first, then moistened with water and finally yeast (dissolved in water) was incorporated. Dough was mixed manually until held together with a non-sticky, smooth and satiny appearance and optimum handling properties. Fermentation was carried out at 29-30 ºC, covered with a damp cloth for 1 hour. Fermented dough samples were stored at -20 ºC until mycotoxin determination.

After fermentation, dough was baked in a muffle furnace (Gallur, Valencia, Spain) at 190, 207, 223, or 240 ºC for 50, 40, 35 and 30 min, respectively (time needed for the bread to be baked at the temperatures used in the procedure). After baking, bread was separated in two portions the inner part and the outside or crust and stored to –20 ºC until their analysis. Bread samples were analyzed in triplicate.

2.3 Extraction and clean-up for OTA

Extraction and clean-up was performed according to the Vicam technical note and suggestions for the analysis of wheat with slight modifications. Seven and half grams of dough or bread finely ground with a laboratory mill (IKA A10, Stauffen, Germany) were extracted with 25 ml of acetonitrile/water (60:40, v/v) in a high speed blender (IKA Ultraturrax T25, Stauffen, Germany) for 5 min. The suspension was centrifuged at 6000 rpm for 6 min at 4 °C (Heraeus Multifuge 1 s-r, Hanau, Germany). Ten milliliters of supernatant were added to 40 ml of pure water. Ten milliliters of this solution were loaded into an immunoaffinity column and passed at a flow rate at 1–2 drop/s. The column was washed with 10 ml PBS and afterwards with 10 ml of pure water and then eluted with 1.5 ml of methanol at a rate of about 1 drop/s in a 4-ml vial. The purified extract was concentrated to dryness at 45 ºC under gentle stream of nitrogen and solved in 0.5 ml of LC mobile phase.

2.4 Liquid chromatographic analysis of OTA

The LC system consisted of a Waters 600 pump, a Waters 717 automatic injector and a Waters 474 scanning fluorescence detector. Millennium 32 software, version 3.05.01 (Waters Co., Milford, MA, USA) was used to control the system and to process signals. The LC conditions were as described by Medina *et al.* [12]. One hundred microliters of the solution were injected into the LC system. Separation was performed on a Gemini C18 column (150 mm×4.6 mm, 5 µm particle size) connected to a Gemini guard column (12.5 mm×4.6 mm, 5 µm particle size) (Phenomenex Inc., Torrance, CA, USA) filled with the same phase. The column was kept at 40 ºC. A mixture of acetonitrile–water–acetic acid (44:56:1, v/v/v) at 1 ml/min flow-rate was used as the mobile phase. Quantification of OTA was performed by measuring its peak area with the help of a calibration curve calculated from OTA standard solutions. Excitation and emission wavelengths were 333 and 460 nm, respectively.

3. Results and discussion

3.1 Fermentation process

Fermentation was carried out at 30ºC for 1 hour. After analysis a decrease in mycotoxin level ranging between 29.8 and 33.5% was observed for the OTA concentration studied. This reduction rate was significant with respect to the initial level ($p < 0.05$).

Although other authors have noted that dough fermentation does not produce a decrease in OTA content [13] it has been reported that fermentation contributes to reduce the contamination with other mycotoxins [14], which could be due to some process happening during fermentation with yeasts. Moreover, for wine it has been pointed out a significant reduction of OTA at the end of alcoholic fermentation during winemaking [15]. However, depending on the yeast strain involved in the fermentation process, there was a difference in the content of OTA in the wines. The percentage of OTA removal during the fermentation was between 46.83% and 52.16% in white wine and between 53.21% and 70.13% in red wine.

3.2 Baking process

To perform the baking process the fermented dough samples were placed into the furnace at different temperature-times combinations to evaluate possible differences in their effects on OTA reduction. Results of removal rate and their precision (averages and standard deviations) appear in Fig 1. In can be observed that, although baking temperature increases, the percentages of OTA reduction are rather similar. ANOVA of the data did not show significant differences ($p < 0.05$) among the average values for OTA reduction rate at the different temperature/time combinations which may be due to the influence of the baking time that was lower when temperature was higher, thus compensating the effect of temperature. Average OTA reduction rate for all OTA spiking levels was 32.9%.

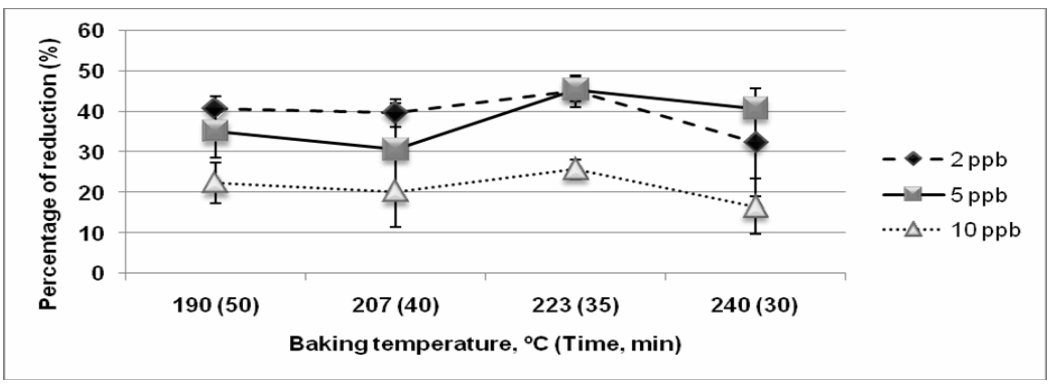

Fig. 1 % OTA reduction (average and standard deviation) during the baking process of bread dough spiked with the mycotoxin at 3 levels (n=3).

Some authors have performed stability assays for OTA on bread baking. Scudamore *et al*. [13] find that only a small fall in concentration happened in bread baked from white or wholemeal flour but they do not report whether this fall was significant. Osborne *et al*. [16] report that no significant loss occurred on baking white or wholemeal flour into bread. However, Boudra *et al*. [17] in their study on the thermal stability of OTA noted that 200ºC kept for 24 min produces 80% reduction in toxin content while this percentage rises up to 94% if time is 48 min. They also find that at 250ºC, 88% of OTA is eliminated in only 16 min.

3.3 Effect of the baking process on the inner and outer part of bread

When the amounts of OTA remaining in the inner and outer part of the bread were compared after bread baking it was observed a greater OTA level decrease on the outer part (Fig. 2). Differences were significant in most cases ($p < 0.05$) independently form the oven temperature/time conditions. Reduction rate was higher on the outside. However, when the concentration of added OTA was 2 ng/g, that statistical difference was not observed but, on average, OTA reduction rate in the outer part of the bread was slightly higher than in the inner part. Difference between reduction in the two zones varied from 4.91% when OTA spiking level was 2 ng/g to 15.4% when OTA spiking level was 5 ng/g. The average difference in reduction ratio was 10.8%. The fact that the inner part of bread takes more time than the surface to reach the furnace temperature can likely explain these results.

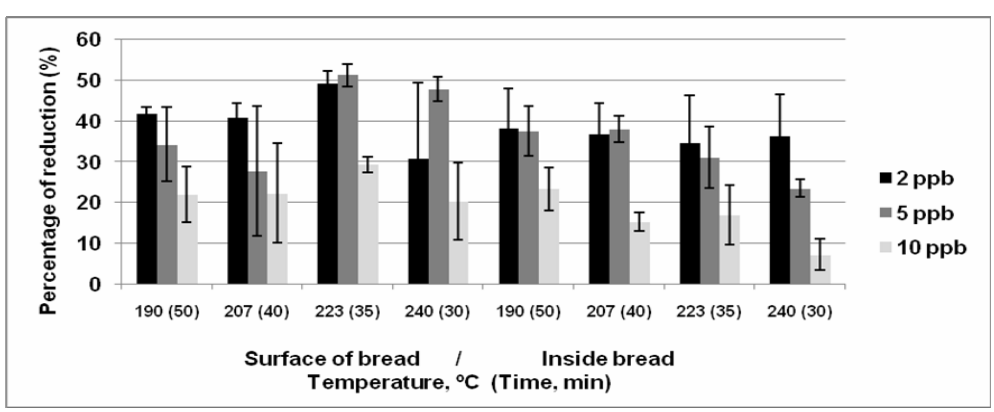

Fig. 2 Average and standard deviation of OTA reduction rate in the inner and outer part of bread made from flour spiked with OTA at 3 levels (2, 5 and 10 ng/g) and baked under different conditions (n=3).

4. Conclusions

During the bread making the level of OTA present at the beginning in the flour is reduced both by fermentation and by further baking inside the furnace. Baking reduction is not very dependent on the usual baking conditions (190-240ºC) because higher temperatures are combined with lower times. Reduction rate of OTA is lower in the inner part of bread probably due to the less time this part undergoes the baking temperature.

Although bread making removes part of this mycotoxin, it cannot eliminate completely the risk of its intake by bread consumption. Thus, it will be necessary to take control measurements aimed to minimize that risk and to keep this food product under concerning regulations.

Acknowledgements The authors wish to thank financial support from the Spanish Government "Ministerio de Educación y Ciencia" (Projects AGL-2004-07549-C05-02/ALI and two research grants) and from the Valencian Government (Project GV04B-111 and a research grant).

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Entrapment by Ca-Alginate Immobilized Yeast Cells for Dried Longan Wine Production

S. Kongruang*, B. Wonganu

Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand, 1518 Piboonsongkram Road, Bangsue, Bangkok, Thailand 10800

*Corresponding author: stk@kmutnb.ac.th Phone: 662-5878259

Two yeast strains, *Saccharomyces bayanus* EC 1118 and *Saccharomyces carlbergenesis* TISTR 5345 were studied for their ability to entrap cells in calcium alginate for dried longan wine production. Immobilized *Saccharomyces bayanus* EC 1118 cells were prepared in three sized-bead diameters, 1.25 mm, 2.50 mm and 4.0 mm to compare with the free cells for the alcohol production. Results showed that 1.25 mm biocatalysts produced the highest alcohol, 9.22 %, while the diameters of 2.5 mm and 3.0 mm yielded 9.12 and 9.00 %. When compared with free cells, immobilized cells produced higher alcohol concentrations. Cell stability levels were tested over the course of fermentation time for the cell leakage to check for repeated utilization. The highest cell leakage was found in 3.0 mm. Size, and lesser amounts were found in 2.5 mm. and 1.25 mm. Sizes with the leakage percentages of 22.22, 12.50, and 1% were found, respectively. Immobilized *Saccharomyces carlbergenesis* TISTR 5345 beads in diameter of 2.50 mm. resulted in 9.10 % alcohol , while yields of 9.00 % and 8.60 % alcohol content yielded from those of beads size 3.0 mm and 1.25 mm bead sizes. Immobilized beads catalyzed the reaction faster and produced higher alcohol production than those of free cells. Leakage percentage of 22.22 %, 12.50 % and < 1 % were found in 4.0 mm, 2.50 mm and 1.25 mm size, respectively. Fermentation kinetics of substrate utilization and alcohol production over a time-course of 14 days were observed. A sensory analysis of the wines was also performed. The physical characteristic of wine color was also reported in the CIELAB system.

Keywords *Saccharomyces bayanus*; *Saccharomyces carlbergenesis*; immobilization; wine; calcium alginate

1. Introduction

Two yeast species *Saccharomyces bayanus* and *Saccharomyces carlbergenesis* are of industrial importance since they are involved in the production process of beverages such as wine, ale and lager beers. In alcoholic beverages fermentation, ethanol is the main product which has long been accomplished in both batch and continuous fermentation throughout the world. Immobilization especially entrapment of cells is the most widely used for fixation of cells in bioprocessing. It is expected that this technique offers advantages such as an increase in cell stability, the possibility of repeated and continuous use of biocatalysts, as well as ease of handing in the recovery process and reducing of the contaminants from the liquid medium. Calcium alginate is commonly used for cell and/or enzyme immobilization since the hydrogel formation occurs under very mild conditions, forms the biocompatibility and has good mechanical strength for many different applications. A comparison between free and immobilized yeast cells was investigated for ethanol production [1-3]. In this study, the effects of bead diameter of immobilized biocatalyst from two yeast strains were determined to find the appropriate immobilized cell size for the efficiency of wine production.

2. Material and Methods

2.1 Inoculum Preparation

Saccharomyces bayanus EC 1118 (Lavin, Danstar ferment AG, Denmark) and *S. carlbergenesis* TISTR 5345 (Bangkok MERCEN) were maintained on Yeast Malt Agar (YMA) at 30 ^{0}C before inoculated on Yeast Malt Broth and incubated for 24 h in a temperature control shaker at 30 ^{0}C. Yeast cells were harvested by centrifugation at 3,000 x g for 10 min at 4 ^{0}C for 10 min. Cells were cleaned with 0.85 % saline solution twice before immobilization. Both free cells and ready to immobilized cells were ready to further steps.

2.2 Cell immobilization

Five milliliters of cell was mixed with an equal volume (1:1 v/v) of 4% (w/v) Na-alginate and then added dropwise to 100 ml of 2 % $CaCl_2$ with a peristaltic pump and pass through syringe with different tip sizes. Cell-alginate polymerization was allowed to occur for 1 h and then was washed with sterile saline solution to remove excess calcium ions and cells. Immobilized cell in large, medium, and small beads (mean diameters, about 1.25 (415 beads), 2.5 (202 beads) and 4 mm (120 beads)) were collected and used as biocatalysts for longan wine production. The experiment was carried out in triplicate.

2.3 Batch fermentation

Commercial available longan fruit was purchased from local market in Thailand. A 50 g longan was boiled with a liter of water. Sample was then filtrated and adjusted to 15 ^{0}Brix, pH 4.0. Medium was then supplemented with $(NH_4)_2SO_4$ to increase nitrogen source. Then 0.2 g/l of potassium metabisufite was added to kill the microbial residues overnight before inoculation. Ethanol productions were carried out in 2000-ml bottles containing 1500ml of filtrated longan juice. Free and immobilized cell in different diameters were added and incubated on shaking incubator at 250 rpm, 30 ^{0}C over two weeks.

2.4 Analytical methods

The total viable numbers of free-cell medium expressed as CFU were determined by the plate count method using YMA. To count the viable number in beads, 3 beads of each diameter were washed with a sterile saline solution and dissolved for 10 min in 30 ml of a sterile 0.1 M sodium citrate. The cultivation of yeast was done at 30 ^{0}C for 48 h. The concentration of reducing sugars was determined by dinitrosalicylic colorimetric assay using glucose as sugar standard. The ethanol concentration was determined by vinometer (Alla, France). Color of the fermentation product was evaluated by colorimeter (Hunter Lab). All determinations were done in triplicate. Statistical analyses were done with SPSS Version 10.

3. Result and Discussion

3.1 Effect of bead diameter by fermentation with immobilized S. bayanus EC 1118 and S. carlbergensis TISTR 5345

During the first week of fermentation using *S. bayanus*, ethanol concentrations of immobilized cells at bead sizes 1.25 mm, 2.50 mm, 4.0 mm.and free-cells were achieved at 5.90, 6.47, 7.30 and 7.631 %, respectively. The highest ethanol production (9.22%) was obtained with cells entrapped in 1.25 mm Ca-alginate beads while 9.12% and 9.00% ethanol was produced with 2.50 mm and 4.0 mm diameter beads. As shown in Figure 1, the increment of bead diameter increased significantly ethanol production over an interval of week ($p = 0.001$). Similar results were obtained with *S.carlbergensis* during the first week of fermentation ($p = 0.008$) as shown in Figure 2. Ethanol concentrations yielded during the first week were 5.10, 5.62, 6.33 and 7.27 %, respectively. In contrast to the second week, the ethanol concentration was not strongly dependent on the bead diameter.

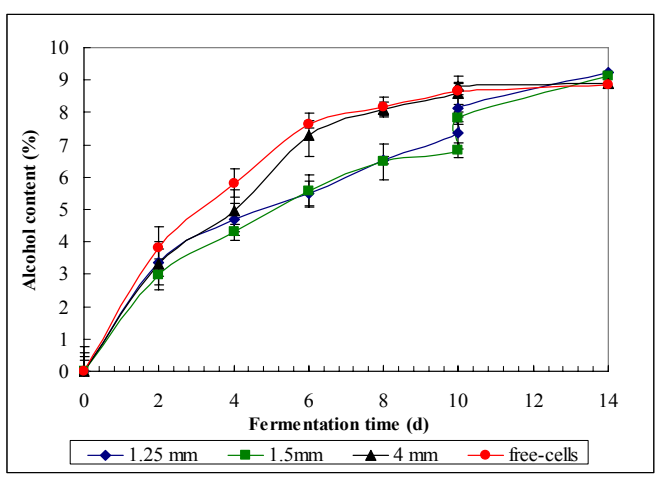

Fig. 1 Time course of alcohol production by *Saccharomyces bayanus* EC 1118

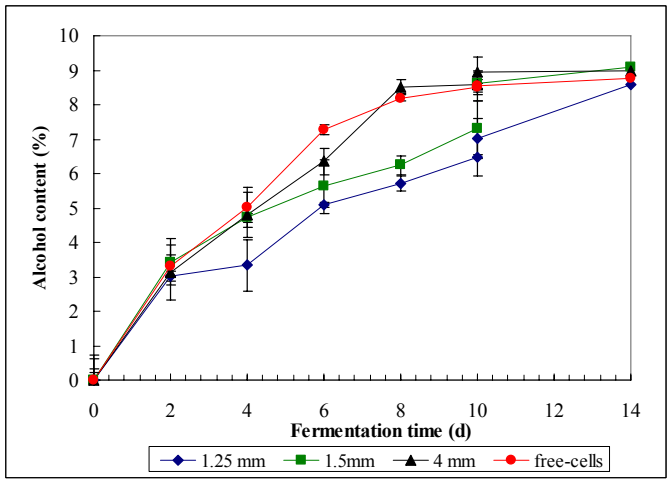

Fig. 2 Time course of alcohol production by *Saccharomyces carlbergenesis* TISTR 5345

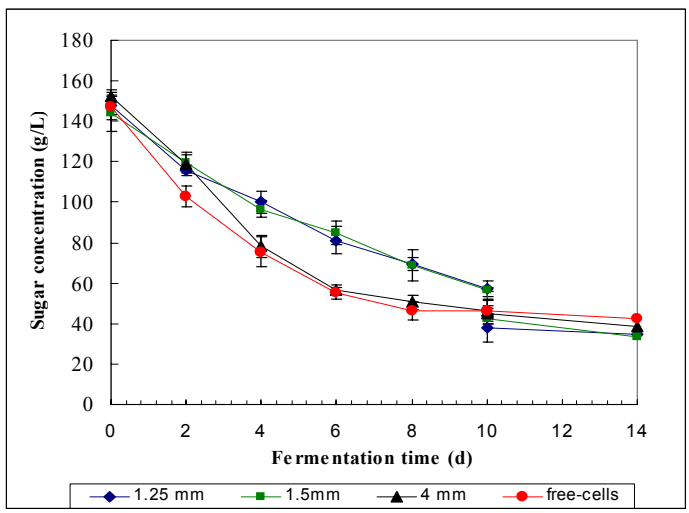

Fig. 3 Time course of substrate utilization by *Saccharomyces bayanus* EC 1118

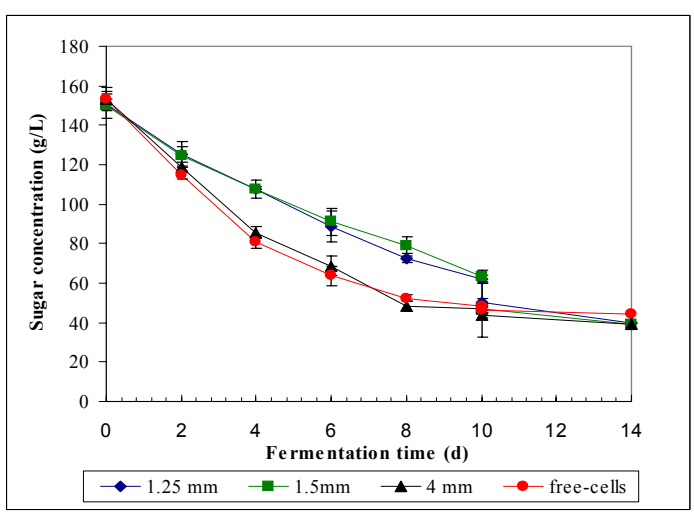

Fig. 4 Time course of substrate utilization by *Saccharomyces carlbergenesis* TISTR 5345

The highest ethanol production (9.10%) was produced with immobilized cells in 2.50 mm diameter. The correspondences of substrate utilization during the alcohol production were also illustrated in Figures 3 and 4 for *S. bayanus* and *S. carbergenesis.*

3.2 Stability of immobilized cells

The influences of the entrapment parameters as bead size and initial cell number on the survival of the yeast cell were quantitatively and systematically determined. Figures.5-7 show the stability comparison of cells in Ca-alginate between *S.bayanus* and *S. carlbergenesis* of different immobilized beads diameter during the course of fermentation. A significant cell loss in 4.0 mm was observed in both strains as 72.29×10^2 cfu/ml and 56.92×10^2 cfu/ml in that order. A bead of 4 mm diameter suffered some breakage, whereas 1.25 and 2.50 suffered to lesser extent. For *S.bayanus*, cell leakages of 96.04×10^1 and 72.50 cfu/ml were found in 1.25 and 1.5 mm, whereas those bead sizes were found only 66.00 and 31.63×10^1 cfu/ml in *S. carlbergenesis.* The survival of cells in smaller bead sizes provided more protection against mechanical mixing while the larger bead diameter can cause a coarseness of gel matrix resulted in insufficient cell holding capacity.

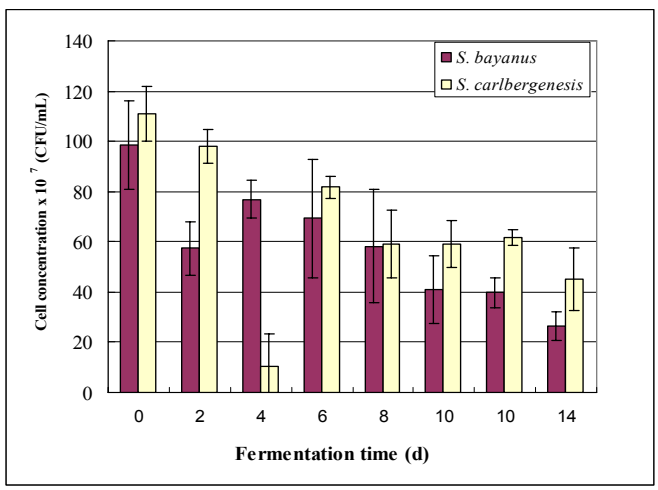

Fig. 5 Comparison of viable cell of *Saccharomyces bayanus* EC 1118 and *Saccharomyces carlbergenesis* TISTR 5345 in 1.25 mm bead size

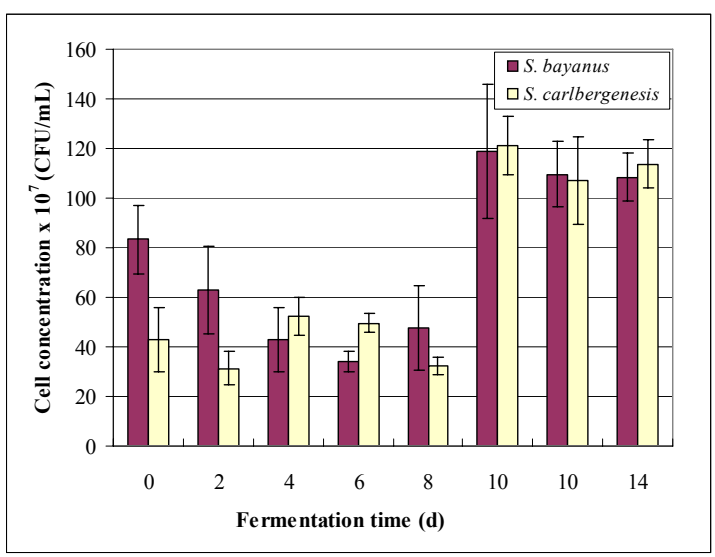

Fig. 6 Comparison of viable cell of *Saccharomyces bayanus* EC 1118 and *Saccharomyces carlbergenesis* TISTR 5345 in 2.50 mm bead size

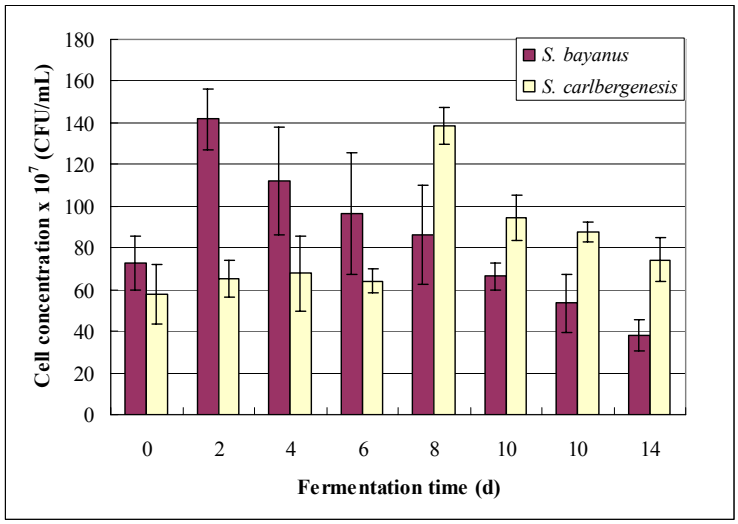

Fig. 7 Comparison of viable cell of *Saccharomyces bayanus* EC 1118 and . *Saccharomyces carlbergenesis* TISTR 5345 in 4.0 mm bead size

3.3 Color appearance

There are no significant difference in color of ethanol produced by *S. bayanus* both free and immobilized cells; however, *S. carlbergenesis* fermentation broth was detected significantly different in color intensity among using different bead sizes ($p<0.0001$) as shown in Table 1.

Table 1 Color intensity of longan wine fermented by *Saccharomyces bayanus* EC 1118 and *Saccharomyces carlbergenesis* TISTR 5345

Strains	Color parameters	Bead size (mm)			Free cell
		1.25	2.50	4.0	
S. bayanus	L*	2.80	2.36	3.42	3.49
	a*	-0.28	-0.33	-0.38	-0.46
	b*	2.03	1.51	2.37	1.90
S. carlbergenesis	L*	5.70	5.41	2.28	2.65
	a*	-0.44	-0.46	-0.33	-0.39
	b*	1.57	1.99	1.25	1.49

4. Conclusions

From this study, it was found that the survival of entrapped wine fermenting yeasts was strongly dependent on bead diameter. Among the techniques for immobilizing living cells, gel entrapment using natural polymer is favored by most researcher for various reasons, including non-toxicity of the matrix, simplicity of immobilization technique, high viability, and productivity of immobilized cells. However, one of the problems is oxygen transfer is often the rate limiting step in a suspended cell culture, and it is more so in an immobilized cell culture. Oxygenation in an immobilized cell culture is one of the major technical problems that remain to be solved. In light of the oxygenation problems, immobilization techniques have been mainly confined to anaerobic processes in which either obligate (strict) anaerobes are employed or only the anaerobic components of the facultative metabolic mechanisms are selectively utilized.

Acknowledgements The support by King Mongkut's Institute of Technology North Bangkok, Bangkok, Thailand is gratefully acknowledged.

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Fungal spoilage in corn

Inês Martins Almeida[1], Marta Franco Marques[2], Ema Torrado[3] and H. Marina Martins[2,*]

[1] Universidade Lusófona de Humanidades e Tecnologia, Departamento de Medicina Veterinária, Campo Grande 376, 1749-024 Lisboa, Portugal
[2] Laboratório Nacional de Investigação Veterinária, Serviço de Micologia, Estrada de Benfica 701, 1549-011 Lisboa, Portugal
[3] Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa, Portugal

[*]Corresponding author: Phone No. +351217115336, E-mail: marina.martins£lniv.min-agricultura.pt

Cereals are economically very important in the food supply systems and imperative as raw material for feed production. Specific competent moulds of the *Aspergillus, Fusarium* and *Penicillium* genera are able to produce mycotoxins that contaminate raw materials, feed or food. In this context were mycologically analysed 37 samples of corn according to official standard procedures (NP-3277-2, 2002). The study revealed that all samples were positives for fungal contamination. The mean contamination level of the corn samples was 3.9 $\log_{10}$ cfu/g, ranging between 1.7 $\log_{10}$ cfu/g up to 4.6 $\log_{10}$ cfu/g. Yeasts were found in 4.6 $\log_{10}$ cfu/g. The most prevalent fungi recovered from corn were *Fusarium* spp., *Penicillium* spp. and *A. flavus,* with mean levels of 4.5 $\log_{10}$ cfu/g, 4.3 $\log_{10}$ cfu/g and 4.0 $\log_{10}$ cfu/g, respectively. The higher frequency of *Fusarium* spp. could be an indicator of the possible presence of *Fusarium* toxins (zearelenone, T-2 toxin and HT-2 toxin, deoxynivalenol, diacetoxyscirpenol and fumonisins), very harmful to animals and humans.

Keywords corn, contamination, *Aspergillus*, *Fusarium*, *Penicillium*

1. Introduction

The increasing worldwide concern about food safety has enhanced interest in fungal infection and subsequent production of mycotoxins in food products. These contaminations with moulds cause deterioration of products and can affect seriously human and animal health. In relation to this, interest is continuously focused on maize because it is one of the most important dietary staple foods in the world [1].

Maize is the most important raw material used in food and feed production. The contamination of this raw material with moulds and mycotoxins is very frequently. This contamination can lead to nutrient losses and can have detrimental effects on animal healthy and production. Because of it, it is necessary to control their mycological quality. Drought, humidity, temperature, insect infestation and rough handling have been suggested as factors which contribute to the presence of fungi and subsequently toxins, in agricultural products, like cereals [2].

Several fungi are associated with maize during pre and post harvest periods, of which the genus *Fusarium, Penicillium* and *Aspergillus* contains important toxigenic species. In Portugal, there are few published reports about occurrence of mycobiota in raw materials, and in this studies, *Fusarium* spp. is the most frequently genus which appears in contaminations of this products. Among other mycotoxins, *Fusarium* moulds, in certain ecological conditions, can produce fumonisins and zearalenone (ZEA). These mycotoxins can cause serious damages in animals healthy like pulmonary oedema in swine or disturbs in the animals reproduction [3].
The intentions of this work are the characterization of mycobiota in corn and determine which are the more frequents moulds in this raw material for elaboration of mixed feed.

2. Materials and methods

2.1 Sampling

A total of thirty seven samples of corn were analysed for determine natural moulds contamination. The samples were randomly collected of different geographic areas of Portugal.

2.2 Mycological analysis

Mycological analysis was performed according official standard procedures (NP-3277-2, 2002; Food Microbiology – General rules for counting moulds and yeasts at 25ºC; Part 2: Reference method).

Ten grams of each sample was added to 90 ml peptone water. From each dilution in peptone water, 1 ml was spread onto plates of Dichloran Rose Bengal Chlortetracycline Agar (DRBC) with 0,25 ml per plate. The plates were incubated at 25ºC, in the upright position, during 5 / 7 days. Each isolated mould colony was observed microscopically for morphological characterization and identification at genus level, compared to descriptions given by Domsch et al. [4]; Barnett and Hunter [5].

3. Results and discussion

Considering a well established mycological quality criteria [6], 45.9% of the corn samples have a reduced Total Mycological Content (TMC) (TMC < 1.5 x 10^4 cfu/g); 16.3% of the total samples haven't presented excessively high fungic contamination (1.5 x 10^4 < TMT < 3.0 x 10^4 cfu/g); 37.8% of samples must be classified as low mycological quality samples, since they presented a high TMC (TMC > 3.0 x 10^4 cfu/g). Considering only the total mycological contents, the results are apprehensive even so, the good quality samples represent the majority. It reveals that subsist a large contamination level of this raw material. If raw materials are not submitted to thermal treatments (physical or chemical), that substantially reduce its microbiological content before being incorporated in feedstuffs, the tendency is to develop this variety of contamination.

It's important to mention that a mycological quality criteria only present a guidance to a mycological survey though, it is extremely important to carry on with determination, interpretation and quantification of fungal contamination levels, as well as evaluating the storage conditions of products and trace the zootechnical profile of the animals that consume feed [7]. Maize samples with good mycological quality, in generality, present a diversified flora, with reduced total and medium fungic contents. They present a lower frequency of potentially toxinegenic moulds, as *Penicillium* spp., *Fusarium* spp., and *Aspergillus* group *glaucus* and *flavus*. Nevertheless, it is necessary to prevent proliferation of this variety of flora. *Aspergillus* and *Penicillium* genera are one of the main responsible for the deterioration of products and stored foods/feed [8]. These groups of moulds, in favourable conditions, can synthesize diverse mycotoxins, like ochratoxins and aflatoxins, whose toxic effects in animals are widely registered [9, 10, 11].

In all analyzed samples the most prevalent genera was *Fusarium*, characteristic from the pasture flora. Its dominant presence denounces that cereals could have been incorrectly manipulated - insufficient drying and precarious conditions of storage - given that *Fusarium* genera needs a great availability in water, to develop itself [12, 13]. It is important to indicate that *Fusarium* spp. includes diverse producing toxigenic lineages in maize and derived products [14]. On the other hand, samples classified as being of reasonable mycological quality, even presenting a considerable contamination level, it was mainly contamination by yeasts. Yeasts don't produce mycotoxins, but the main inconvenience of the presence of these unicellular fungal in feed, is that they can originate modifications in the sensorial properties of commodities, conducing to a decrease in their quality.
All 37 maize samples showed fungic contamination (100%). It was proved that the predominating fungi genera in the analyzed samples were *Fusarium* (86.5%), followed by *Penicillium* (67.6%) and *A. flavus* species (62.2%), accordingly to Table 1.

The average samples content (M) was 7.3 x 10^3 cfu/g. The results obtained in this study, even with a modest significant data, are in accordance with some authors who have published similar works on stored maize, identifying *Fusarium*, *Penicillium* and *Aspergillus* genera as the primarily contaminants in this cereal [2, 8, 15].
Although less frequents the genera *Mucor* (43.2%), *A.glaucus* (43.2%) e *A. candidus* (40.5%) were also found (Figure 1). In 97.3% of the samples revealed the yeasts presence, with a contamination level that ranged between 1.0 x 10^1 e 1.0 x 10^6 yeasts /g.

Table 1- Mycological mean levels of different genera (cfu /g and Log_{10})

Fungi	**Average (cfu/g)**	**Average (log_{10} cfu/g)**
Yeasts	3.8×10^4	4.6
Asp. Flavus	1.0×10^4	4.0
Asp. fumigatus	1.1×10^3	3.0
Asp. Níger	1.0×10^3	3.0
Asp. terreus	6.7×10^3	3.8
Asp. candidus	1.0×10^4	4.0
Asp. glaucus	1.7×10^4	4.2
Asp. flavipes	6.4×10^2	2.8
Asp. ochraceus	7.5×10^1	1.9
Asp. versicolor	3.6×10^2	2.6
Acremonium spp.	2.4×10^3	3.4
Cladosporium spp.	6.7×10^3	3.8
Penicillium spp.	2.1×10^4	4.3
Mucor spp.	8.8×10^2	2.9
Trichoderma spp.	4.8×10^1	1.7
Fusarium spp.	3.5×10^4	4.5
Rhizopus spp.	2.1×10^2	2.3
Absidia spp.	1.5×10^3	3.2
Scopulariopsis spp.	8.1×10^2	2.9
Verticillium spp.	1.1×10^3	3.0
Alternaria spp.	1.0×10^2	2.0
Mean Total Content	7.3×10^3	3.9

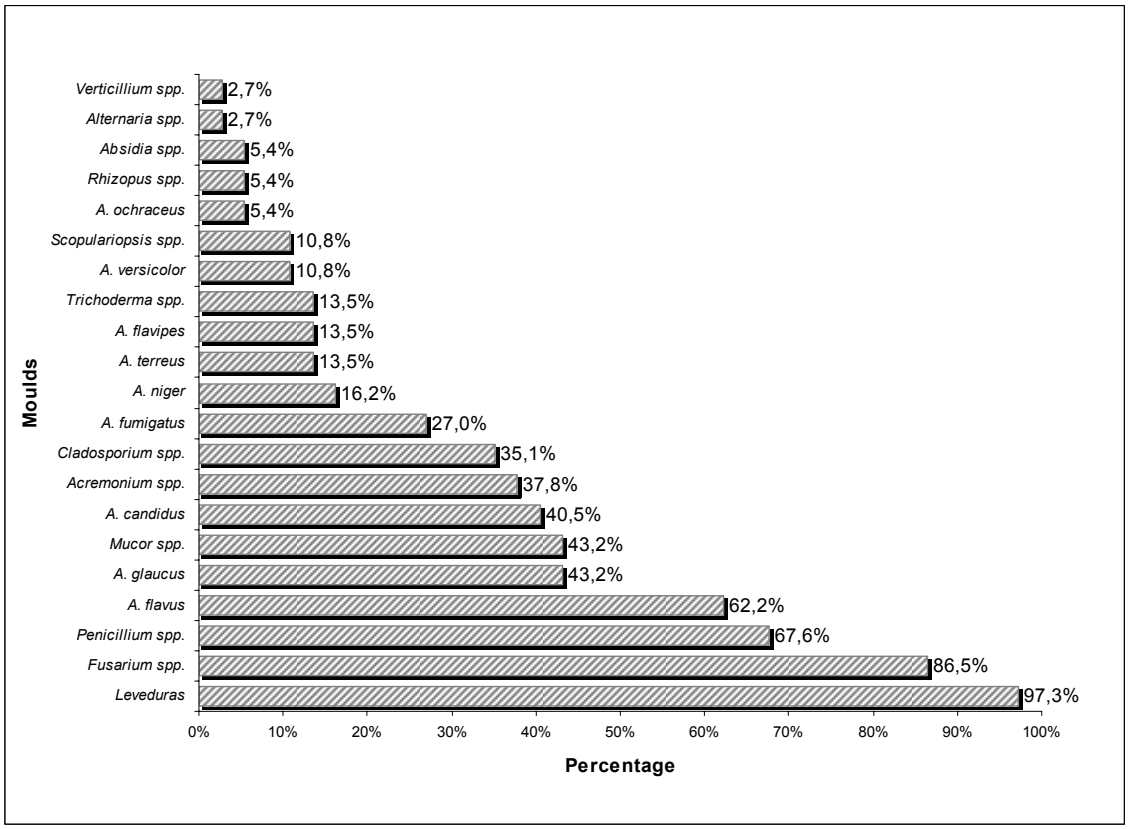

Figure 1 – Frequency of different genera and species found in positive samples.

When mycological analysis of raw materials denounces high fungic contaminations, mainly from potentially toxigenic moulds, it is advisable to quantify mycotoxins levels and, depending on the result, to consider its use in the production of feed and food. It is important to judge some important factors, such as: category of mycotoxins detected; contamination level; incorporation percentage of the raw material, in food and feedstuffs, among others. However, it has necessary to have in attention that the fungal contamination can be "masked", due to use of antifungic products or other technological treatments.

Acknowledgments The authors would like to acknowledge to Candida Camejo and Anabela Ramos for their collaboration in this work.

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Isolation, phenotypic and genotypic characterization of quinolone-resistant *Salmonella enterica* strains isolated from foods and water

A. Jiménez[1], Y. Moreno[1], A. González[1], D. Tomás[2], J. L. Alonso[3] and M. A. Ferrús[1]

[1] Departamento de Biotecnología, Universidad Politécnica de Valencia, 46022 Valencia, Spain
[2] ainia Centro Tecnológico, 46980 Paterna, Spain
[3] Instituto de Ingeniería del Agua y Medio Ambiente, Universidad Politécnica de Valencia, 46022, Valencia, Spain

[*]Corresponding author: Phone: +34 96 387 7423, Fax: +34 96 387 9429, e-mail: mferrus@btc.upv.es

120 Salmonella strains were isolated from river, sewage, sea water and food sources. Biochemical profiles, serotype and susceptibility to 12 antibiotics was determined. Quinolone-resistant strains were analysed by PCR-RFLP and sequencing analysis. A total of 23 different serovars were identified, being S. enterica serovar Hadar predominant (22 %). Salmonella Enteritidis was accounted for only 2.3% of the strains. A high proportion (87%) of the isolates was resistant to at least one antimicrobial agent, and 52% were multidrug-resistant. Twenty four of the strains showed reduced ciprofloxacin susceptibility and were resistant to nalidixic acid. All of them presented at least one point mutation at either Ser83 or Asp87 in the QRDR region of the gyrA gene. This study shows high rates of multidrug and quinolones resistant Salmonella strains in foods and environment, what indicates an increased risk of human acquisition via the food chain and can become a public health hazard.

Keywords Salmonella; quinolone resistance; food; water

1. Introduction

Salmonella is a large genus of bacteria that includes more than 2400 serotypes. Salmonellosis is the most common food and waterborne transmitted disease, being human and animal excreta the most important sources of *Salmonella* in the environment [1].

Most typing methods are being using for epidemiological studies of this genus. Serotyping is a useful tool for epidemiological surveillance of *Salmonella* species and offers a precise and reliable method for differentiating isolated strains. In Spain, serotypes most frequently isolated from foods and environment are Enteritidis, Typhimurium and Hadar [2], being *S.* Typhimurium and *S.* Enteritidis the most important serotypes causing clinical cases [3].

Detection of *Salmonella* spp. showing decreased susceptibility to quinolones has become an important global issue, as they are increasingly isolated from animals and food products [4]. Probably both human and veterinary uses have significantly contributed to the emergence of quinolones resistant strains of *Salmonella*. This is a public health concern matter, since it is often accompanied by multiresistance to other important therapeutic antibiotic classes. Moreover, the emergence of resistance to extended-spectrum cephalosporins associated with quinolones resistance are associated with treatment failure and even excess mortality and morbidity [5].

Although quinolones resistance can involve a variety of different mechanisms, in most Gram-negative bacteria it is mainly conferred by point mutations in the *gyrA* gene coding for the A subunit of DNA-gyrase [6]. Resistance mutations of *gyrA* have been clustered in a region of the gene product between amino acids 67 and 106, termed the quinolone resistance-determining region (QRDR) [7]. Amino acid changes at Ser-83 or at Asp-87 are the most frequently observed in nalidixic acid-resistant strains. Double mutations at both residues S83 and A87 have been identified in fluoroquinolone-resistant *Salmonella* spp. Alterations at residue Gly-81 have also been identified in low level quinolone-resistant spontaneous mutants of *S.* Typhimurium. Topoisomerase IV is a secondary target for quinolones, and mutations in the genes *parC* and *parE* at positions, equivalent to those identified in *gyrA* and *gyrB*, participate in high-level resistance to quinolones. The contribution of *gyrB* mutations to quinolone resistance is still unclear [6].

Over the last decades, several molecular techniques have been applied to detect the mutations conferring quinolones resistance to *Salmonella* clinical and food borne strains [8]. Restriction fragment length polymorphism analysis of PCR-amplified DNA fragments (PCR-RFLP) is one of the most interesting methods used, as it is fast, easy to perform and cost effective [9].

So, the aim of this study was to determine the epidemiological, phenotypic, and genotypic characteristics of *S. enterica* present, either in the environment or in food products in our geographical area (Comunidad Valenciana, Spain). We focused on the prevalence of quinolones resistant isolates, and the detection of mutations in QRDR of *gyr*A gene.

2. Material and Methods

A total of 120 *Salmonella* strains were isolated from different environmental sources: Thirty strains were obtained from sea water and 10 from sewage; Seventy strains were isolated from food samples, 55 of them from chicken and meat products. Ten human isolates were obtained from faeces of different healthy excreters (carriers). *Salmonella enterica* NCTC 12117 was used as a reference strain.

2.1 Phenotypic characterization

The isolates were purified and characterized by morphology, Gram stain, oxidase, catalase and API-20E identification system (Biomérieux, France). Serotyping was performed at Centro Nacional de Microbiología (Instituto de Salud Carlos III, Spain).

Antibiotic resistance was determined by Disk diffusion tests (Oxoid, Basingstoke), according to the NCCLS guidelines [10]. Susceptibility to amikacin, ampicillin, amoxicillin/clavulanate, sulfamethoxazole-trimethoprim, ceftriaxone, ciprofloxacin, chloramphenicol, carbenicillin, gentamicin, nalidixic acid, tetracycline and cephalothin was determined. *E. coli* ATCC 25922 was used as a control.

When resistance or intermediate susceptibility to quinolones was observed, results were confirmed by E-test (AB BIODISK, Sweden). According to NCCLS guidelines, the breakpoint for ciprofloxacin resistance was established at MIC $\geq$ 4 μg/mL and for nalidixic acid at $\geq$ 16 μg/mL.

2.2 DNA isolation and PCR-RFLP analysis

Chromosomal DNA was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method [11] and DNA pellets were resuspended in nuclease-free water. DNA concentration was determined by spectrophotometer absorbance readings at 230, 260 and 280 nm, and integrity of DNA was tested running the DNA on 1% (w/v) agarose (Pronadisa, Madrid, Spain) gel electrophoresis. Templates were stored at -20 °C until use.

Primers *gyr*AP1 (5'-TACCGTCATAGTTATCCACG-3') and *gyr*AP2 (5'-GTACTTTACGCCATGAACGT-3') were used to amplify a 313-bp PCR fragment of the QRDR from *gyr*A gene of all the *Salmonella* strains [9]. PCR was performed in 50 μl of a mixture containing 10 μl of template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and 2 U of Taq polymerase.

The amplification consisted of an initial denaturation step at 95ºC for 5 min followed by 30 cycles (denaturation at 94ºC for 1 min, specific primer annealing at 57°C for 1 min and extension at 72ºC for 1 min), ending with a final extension at 72ºC for 10 min. DNA templates from reference strain were used as positive controls. In addition, negative controls in which DNA was replaced with sterile distilled water was also included. All the reagents used in the PCR reactions (Taq polymerase, dNTP and $MgCl_2$) were provided by Roche Diagnostics (Indianapolis). The primers employed were prepared by TIB MOLBIOL (Germany).

PCR products were detected by electrophoresis on 1.2% (wt/vol) agarose gel in 1× Tris-Acetate-EDTA (TAE) buffer at 100 V for about 60 min, and visualized by staining with ethidium bromide (0.5 μg/mL) and ultraviolet transillumination (UV). As a molecular marker, the GeneRuler 100-bp DNA Ladder Plus (Roche) was used.

For restriction analysis, PCR products (15 μl) were digested with 10 U of restriction enzyme *Hinf*I (Roche Diagnostics) in a final volume of 20 μl at 37 °C for 2 h. The reaction was stopped by adding 3 μl of stop-mix solution (50 mM EDTA, 0.3% Ficoll, 0.3% bromophenol blue).

Restriction fragments were separated on 2% (wt/vol) agarose gel electrophoresis in TAE 1× buffer at 90V for 3 h and visualized after staining with ethidium bromide and UV transillumination. GeneRuler 50-bp DNA Ladder Plus (MBI Fermentas, Canada) was used as a standard for molecular size determination.

In order to assess reproducibility of PCR-RFLP analysis, all the strains were analysed at least two times in different experiments.

2.3 Sequencing of QRDR region of *gyr*A gene

Quinolone-resistant strains were analysed in order to determine the presence of mutations in the quinolone resistance determining region (QRDR) of *gyr*A gene by sequencing analysis. Ten quinolone susceptible strains were sequenced too for comparison of results.

PCR products were purified with a Qiaquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. Both DNA strands were sequenced by Sistemas Genomicos S. L. (Valencia, Spain). The sequences were analysed by using the BLAST software (http://www.ncbi.nlm.nih.gov/blast/).

3. Results and Discussion

A total of 23 different serovars were identified, most of them including less than 5 % of strains. As shown in Table 1, *S. enterica* serovar Hadar was predominant (22 %) followed by S. Typhimurium, S. Livingstone and S. Anatum. *Salmonella* Enteritidis, a frequently clinical reported serotype [12], was accounted for only 2.3% of the non-clinical strains.

Table 1 Distribution of the most prevalent *Salmonella* serotypes, according to the origin of the strains

Serotype	Number of strains				
	Sea Water	Seawage	Human	Foods	TOTAL
S. Hadar	0	1	4	22	27 (22 %)
S. Typhimurium	1	2	0	15	18 (15 %)
S. Livingstone	1	0	0	13	14 (12 %)
S. Anatum	9	0	0	1	10 (8 %)

All the strains were susceptible to Amikacine, Gentamicine and Ceftriaxone. A high proportion (87%) of the isolates was resistant to at least one antimicrobial agent, being 52% multidrug-resistant. These results are higher to those found by other authors in Spain [13]. Almost all strains showed a high sensitivity to ciprofloxacin ($\leq$ 0.1 μg/mL). Twenty four of the strains (20%) were resistant to nalidixic acid (MIC > 32 μg/mL). One strain was isolated from a human carrier, while the rest of them were obtained from foods, being twenty strains isolated from chicken and meat products. Twenty three of the nalidixic acid-resistant strains were resistant to other antibiotics, while only one strain was resistant exclusively to nalidixic acid. Although all the nalidixic acid resistant strains showed reduced ciprofloxacin susceptibility ($\leq$ 0.75 μg/mL), no isolate was resistant to this antibiotic.

All the nalidixic acid-resistant strains presented at least one point mutation at either Ser83 or Asp87 in the QRDR region of the *gyr*A gene, confirming previous works [14]. No mutation was observed at codon Gly81. The most frequent mutation was A87 GAC-AAC (Asp/Asn; 18 strains, 75%). Three strains showed A87 GAC-TAC (Asp/Tyr), 2 strains showed S83 TCC-TAC (Ser/Tyr) and one strain showed S83 TCC-TTC (Ser/Phe). No one of the quinolone-susceptible strains analysed showed any mutation at QRDR region.

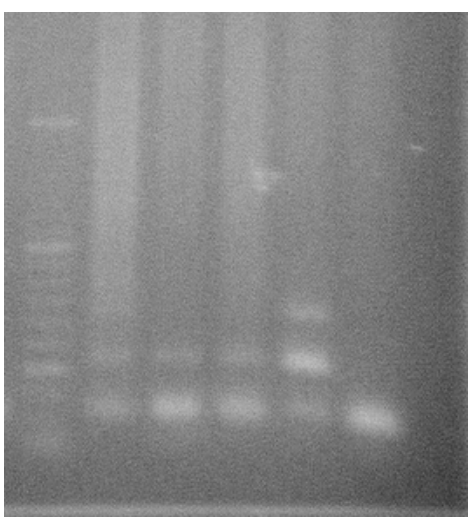

Fig. 1 PCR-RFLP patterns of quinolones resistant and susceptible *Salmonella* strains: Lane 1: Molecular size marker; Lanes 2-5: nalidixic acid-resistant strains; Lane 6: nalidixic acid-susceptible strain

Digestion with restriction enzyme *Hin*f I yielded a specific pattern for the strains presenting mutations at codon S83, as expected [9]. However, PCR-RFLP was not useful to identify quinolone-resistant strains, as most of the strains presented mutation at codon A87, which is not recognized by this enzyme. Moreover, as Figure 1 shows, the presence of faint bands in all the restriction patterns made the interpretation of band profiles very difficult to perform.

4. Conclusions

This study shows high rates of multidrug and quinolones resistant Salmonella strains in foods and environment, what indicates an increased risk of human acquisition via the food chain and can become a public health hazard.

Quinolone-resistance was always associated to one mutation in the QRDR region of the gyrA gene. Hinf1 PCR-RLFP is not useful to detect all the resistant strains when used alone. Sequencing of the QRDR region of the gyrA gene offers a great quantity of information on the molecular mechanism of this resistance.

Acknowledgements This work was supported by Ministerio de Ciencia y Tecnología, Spain (Research Project AGL2005-07776-C03-01, national and FEDER funds).

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Microbiology stability of wine from Castilla la Mancha

M. Arévalo-Villena*, N. Barrajón-Simancas, E. Cerdeño-Gómez, A. Briones

Departamento de Química Analítica y Tecnología de Alimentos, Facultad de Ciencias Químicas Universidad de Castilla-La Mancha, 13071 Cuidad Real, España

*Corresponding author: email: Maria.Arevalo@uclm.es

Castilla la Mancha is a very important wine producer region in Spain. The obtaining of a quality product is each day more important, being one of the principal factors the microbiological stability in wine finished. The objective of the present work was to study the microbiological stability of different kind of wines from three D.O. la Mancha wineries. 17 samples, both white and red wines, were taken at the end of last vineyard being non centrifuged and centrifuged wines, filtered, from barrels and already bottled. YPD agar was used for growing of yeasts. Non *Saccharomyces* species were isolated from YPD plates by replicate platting using lysine agar. In the case of bacteria, there was no growth; neither lactic acid nor acetic when the samples were incubated on Rogosa and EYP and GYC agar plates. Nevertheless 13 of 17 samples presented yeasts contamination. Only two wines contained non *Saccharomyces* yeasts, which were isolated and identified by PCR/ RFLP. Mitochondrial DNA analysis technique was utilized to identify all *Saccharomyces* strains which allowed to know if yeasts found were the same than the starter used for inoculation. Five different *Saccharomyces cerevisiae* strains were found in semi fermented samples; being biodiversity in other wines lower. In wines from barrels, there was a predominant strain presented at 90 % of cases, which indicate that it is a strain adapted to aging process.

Keywords Microbiological stability, biodiversity, wine yeasts, lactic and acetic bacteria.

1. Introduction

The winemaking process includes multiple stages at which microbial spoilage can occur, altering the quality and hygienic status of the wine and rendering it unacceptable. The major spoilage organisms include species and strains of the yeast genera *Brettanomyces, Candida, Hanseniaspora, Pichia, Zygosaccharomyces*, etc., the lactic acid bacterial genera *Lactobacillus, Leuconostoc, Pediococcus*, etc. and the acetic acid bacterial genera *Acetobacter* and *Gluconobacter* [8]. The faults caused include bitterness and off-flavours (mousiness, ester taint, phenolic, vinegary, buttery, geranium tone among others) and visual problems such as turbidity, viscosity, sediment and film formation [2], [9]. These spoilage organisms can also affect the wholesomeness of wine by producing biogenic amines and precursors of ethyl carbamate [1], [10]. The judicious use of chemical preservatives such as sulphur dioxide (SO_2) during winemaking process decreases the risk of microbial spoilage, but resistance strains vary considerably.

Wine spoilage microbes are those found at the wrong place and the wrong time, including microorganisms which are normally desiderable and contribute to the quality of the end product. The winemaking process is a complex ecological niche where the biochemistry and interaction of yeasts, bacteria, fungi and their viruses play a pivotal role in the final product.

There are three stages at which microorganisms can enter the winemaking process and exert an influencing effect on the quality of the end product [4]. The first one involves the raw material, since grapes are in direct contact with the winery equipment (crushers, presses, tanks, pipes, pumps, filtration units, etc.). The second stage of spoilage may occur during fermentation [6]. At this point the grape juice contains the natural flora of the grapes along with the flora harboured by the wine cellar and its equipment. The composition of the grape juice (high sugar and acid content, and low pH) and the addition of sulphur dioxide (SO_2) to the juice exert selective pressure on the development of yeasts and bacteria during the process. *Saccharomyces cerevisiae* is the dominant yeast during fermentation, and the increase in ethanol concentrations further suppresses the development of yeasts belonging to the genera *Candida, Hanseniaspora, Kloeckera* and *Metschnikowia*, and less frequently *Kluyveromyces* and *Pichia*. These non- *Saccharomyces* yeasts are ethanol-sensitive and die off as soon as the ethanol concentration starts to increase during the fermentation process, but with numbers as high as 10^6-10^7 cfu/mL before death, they significally influence the composition of the wine. pH is a crucial factor at this stage [12]. At a wine pH > 3.6 the growth of lactic acid bacteria (LAB), especially *Lactobacillus*, *Leuconostoc* and *Pediococcus* spp., as well as AAB is enhanced, and this may be detrimental to the quality of the wine. The third stage at which the product may be susceptible to spoilage is post-fermentation. Here, spoilage may occur in the bottle or during storage in oak barrels [11], [7].

La Mancha is the largest wine-producing region in Spain in terms of surface area, producing over 50% of the country's table wine. The microbiological control is a very important fact, not only for economic reasons but also with regard to quality, particularly since the numerous cooperatives and wineries in this region process millions of litres of must every year.

For this reason, the aim of this study was to know the viable microorganisms present in finished and aged wines and to establish if there is relationship between the starter used in winemaking and the wild *Saccharomyces* biodiversity.

2. Material and Methods

2.1. Sampling

The study was done during 2006 vintage in three different wineries from Castilla la Mancha. 100 mL of each sample was taken under sterile conditions (Table 1).

Table 1. Characteristics and code of the samples

WINE	*RED / WHITE*		CODE
Semifermented	white	before centrifugation (2006)	B1
	white	after centrifugation (2006)	B2
	red	before centrifugation (2006)	T1
	red	after centrifugation (2006)	T2
Finished not botteled	red	after fine filtration (2006)	T3
	white	after fine filtration (2006)	B3
	red	inoculated fermentation (2005)	T5
	white	spontaneous fermentation, jar (2006)	B4
	white	spontaneous fermentation, jar (2005)	B5
	white	inoculated fermentation, jar	B6
	white	spontaneous fermentation, jar (2006)	B7
Botteled	red	botteled (2002)	T9
	white	botteled (2006)	B8
	red	aged botteled	T6
Barrel	red	oak barrel (2004)	T4
	red	barrel (2005)	T7
	red	barrel (2006)	T8

2.2. Microbiological analysis

All samples or serial dilutions were seeded in agar plates using different media depending on microorganisms found. In cases where there was not any growth SELMED broth was used during 48 hours with agitation to revitalize the sample.

For yeasts isolation each sample was seeded in YPD agar (10 % yeast extract, 20 % glucose, 20 % peptone and 20 % agar) added with tetracycline for bacteria inhibition during 24/48 hours at 30 ºC. Non *Saccharomyces* were distinguished from *Saccharomyces* yeasts by replicate platting on Lysine agar. The samples were also seeded in Rogosa agar (OXOID) plates with ciclohexymide (0.01%) and kanamycin (0. 25 %) for yeasts and acetic acid bacteria inhibition respectively and incubated under anaerobic conditions during 48/72 hours first and then in aerobic conditions three more days always at 30 ºC. Finally EYP (2% ethanol, 1% yeast extract, 0.5 % peptone, 1.5 % agar, 2.2 % bromocresol green) and GYC (5 % glucose, 1 % yeast extract, 3 %& calcium carbonate, 1.5 % agar) agar were used for acetic bacteria isolation during 72 hours at 30 ºC.

2.3. *Saccharomyces* yeasts characterization

Ten *Saccharomyces* isolates from each sample were chosen and restriction analysis of their mitochondrial DNA was done for their characterization by Querol *et al*. (1992) method [5]. DNA (10 μL) was digested with *Hinf*I endonuclease (Boehringer Mannheim). Restriction fragments were separated by electrophoresis on 1.5% agarose gel with added ethidium bromide (0.5 μg mL^{-1}). Gels were visualized in a UV transilluminator and processed using the GeneFlash gel documentation system.

2.4. Non-*Saccharomyces* yeasts Identification

Ten non-*Saccharomyces* isolates from each sample were chosen for identification by PCR-RFLP (Polymerase Chain Reaction/ Restriction Fragment Length Polymorphism) using ITS primers amplifying variable and intergene regions (ITS1 and ITS4) of the 5.8S rDNA gene. For species identification of yeasts, amplification products were digested with restriction enzymes *Hinf*I, *Cof*I and *Hae*III (Boehringer Mannheim GmbH).

Amplification products and restriction fragments were separated by electrophoresis on 1.5 % agarose gel (w/v) with added ethidium bromide (0.5 μg mL^{-1}) [3].

3. Results

3.1. Microbiological analysis

Table 2 shows the microbiological counts of different wines analysed. There was not presence of lactic or acetic bacteria, which means that they are bacteriologically stable wines although 76% of the samples contained yeasts.

Table 2. Yeast Counts (cfu/ml) in some wines from La Mancha region

WINE	*Yeast Counts (cfu/ml)*	Saccharomyces	Non Saccharomyces
Semifermented			
B1	**570.000**	**yes**	**no**
B2	**680.000**	**yes**	**no**
T1	**1.390.000**	**yes**	**no**
T2	**1.110.000**	**yes**	**no**

Finished not bottled			
T3	**1.350.000**	**yes**	**yes**
B3	**2.900.000**	**yes**	**yes**
T5	**850.000**	**yes**	**no**
B4	**23.000**	**yes**	**no**
B5	**nd**[a]	**-**	**-**
B6	**nd**	**-**	**-**
B7	**13.900**	**yes**	**no**
Bottled			
T9	**nd**	**-**	**-**
B8	**nd**	**-**	**-**
T6	**380**	**yes**	**no**
Barrel			
T4	**86.000**	**yes**	**no**
T7	**11.600.000**	**yes**	**no**
T8	**2800**	**yes**	**no**

[a]nd: no detected

Samples B1, B2, T1 and T2 were semifermented products, white and red, before and after centrifugation. As results show, the centrifugation process was not efficient 100 %, since there is almost not decrease of yeast population form samples 1 to 2.

With regard to the finished but not bottled wines, those filtered with diatomaceous earth (T3 and B3) presented around one million of yeasts, *Saccharomyces* and non *Saccharomyces*, which after PCR-RFLP study, were species of *Kluyveromyces bacillisporus* (14.5 %), *Pichia membranaefaciens* (35.5 %) and *Torulaspora delbrueckii* (50 %).

Samples B4 and B7 fermented in jar during vintage 2006 had around 10^4 cfu/ml, which is a normal value in wines when the fermentation is finished. Nevertheless, wines B5 and B6 elaborated in 2005 did not have any microorganisms, even using Selmed media to enrich them.

In bottled wines, the direct seed on YPD agar of T6 did not show any growth, but when Selmed medium was used, there was presence of *Saccharomyces* although in a very low level. The rest of the wines did not have any yeast or bacteria, even when the seed was not direct.

In sample T4, from oak barrel, there was less than 10^5 cfu/ml, although it seems a high level, all yeasts found were *Saccharomyces* and it is difficult for them to produce refermentation process since there is not residual sugar. However, in sample T7, also aged in oak barrel, there was a too high number of yeast, very similar to those found during a normal alcoholic fermentation, so that there could be a refermentation process in the barrel.

3.2. *Saccharomyces* yeasts characterization

To know the *Saccharomyces* biodiversity in finished wines and if these strains were the same as the one used as commercial starter, was consider also very interesting. Ten isolated *Saccharomyces* from the 13 samples with yeasts presence were chosen for restriction analysis of their mitochondrial DNA.

Figure 1 shows the percentage of different strains found in each sample.

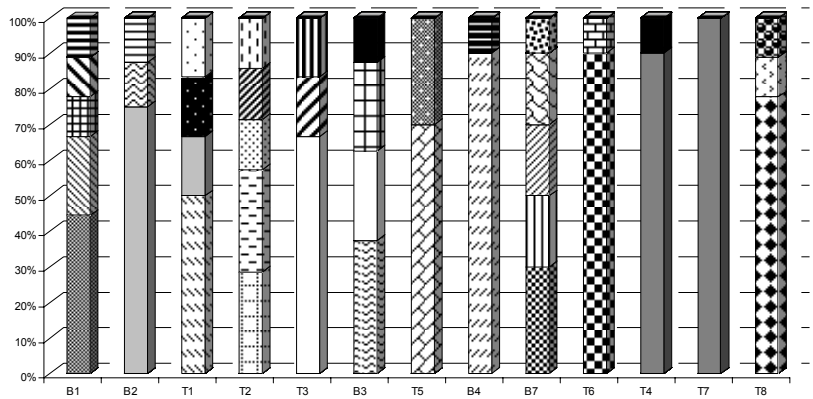

Figure 1. *Saccharomyces* biodiversity in semifermented, finished and aged wines (each strain is represented by a different draw)

Semifermented wines presented a high biodiversity, there being some dominant yeasts. The *Saccharomyces* found were different to the starter used in the winery, only 10 % of isolates from B1, was one of the commercial yeast utilized.

The biodiversity versus kind of wine and vintage shows that in semifermented wines, there is more variability of strain, up to five different, which makes sense since their fermentations were stopped in the middle of the process, when a mix of different strains are present in the fermentation. One more time, it can be observed that the centrifugation process does not reduce very much the number of strains.

In wine B3 and T3, the biodiversity was lower and 60 % of isolates were similar to one starter used in the winery.

With regard to the fermentations done in jar, in sample B4 (vintage 2006) there was a clear dominant strain, nevertheless in the other one (B7) it could be found four different profiles.

In oak barrel aged wines (T4, T7 and T8), there was some predominant strains (more than 80 %) that could come from the own barrel or from the wine. The same happens with T6. All of them are aged wines and the isolated strains are very well adapted to the conditions, normally extremes due to high alcohol concentration and low residual sugar level.

4. Conclusions

In this study it is noticed that some yeasts can remain along the time in finished wines. These microorganisms could cause problems in wines if during storage, they found favourable conditions (temperature, aeration…). This fact shows the need for rigorous microbiological control in cellars to assure the biological stability of wines even when bottling is already done.

On the other hand it can be observed that there is *Saccharomyces* biodiversity in final wines since there are diverse strains which are also different from the starter used to inoculate.

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Occurrence of mycobiota in swine feed

Inês Martins Almeida[1], Marta Franco Marques[2], Ema Torrado[3] and H. Marina Martins[2,*]

[1] Universidade Lusófona de Humanidades e Tecnologia, Departamento de Medicina Veterinária, Campo Grande 376, 1749-024 Lisboa, Portugal

[2] Laboratório Nacional de Investigação Veterinária, Serviço de Micologia, Estrada de Benfica 701, 1549-011 Lisboa, Portugal

[3] Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa, Portugal

[*]Correponding author: Phone No. +351217115336, E-mail:marina.martins@lniv.min-agricultura.pt

Moulds and yeasts are frequently referred as microbial contaminants of feed meals used in intensive animal production. Most of the sanitary risks that are present in milk, eggs and meat are related with the safety of animal feeds. In this study, 75 samples of swine feed, being 10 feed meals and 65 granulated, were tested for mycological characterisation, using conventional methods (NP-3277-2; 2002). Only two granulated feed were negative (2.7%). Out of 75 samples, 73 (97.3%) were positive. Mean count of fungi has been 6.6×10^2 cfu/g ranging from 2.7×10^1 to 2.7×10^3 cfu/g; yeasts were present in 69.9% of the positive samples. Potential toxigenic moulds (*Fusarium* spp., *Aspergillus flavus* and *Penicillium* spp.) were present in all the positive samples with mean levels of 3.2 $\log_{10}$ cfu/g, 2.8 $\log_{10}$ cfu/g and 3.0 $\log_{10}$ cfu/g, respectively. Other genera found were *Phoma, Rhizopus* and *Paecillomyces*, with low levels of contamination (32.9%, 35.6% and 47.9%, respectively). It was concluded that the levels and frequency of mycobiota contamination are decreasing judging the results obtained in the last ten years, in Portugal.

Keywords moulds, yeast, feed

1. Introduction

In Portugal, commercial feedstuffs are an important component in modern animal husbandry. Information about *fungi* associated with food and feeds is important to assess the risk of mycotoxins contamination. Mycotoxins are secondary metabolites that have adverse effects on human, animals and crops, resulting in illness and economic losses [1]. Swine feed are prepared with vegetables, cereal grains (maize, sorghum and rice), fats, vitamins and minerals. During its manufacturing, feed can be contaminated with mould spores [2]. The most commonly filamentous moulds found in stored cereal grains and feeds are *Aspergillus, Penicillium* and *Fusarium* species; they are ubiquitous, can cause feed spoilage and biodeterioration, and are capable to produce many different mycotoxins. *Aspergillus* species (*A. flavus, A. parasiticus, A. nomius, A. fumigatus, A.. versicolor* and *A. ochraceus)* are some of the more common toxigenic species [3]. *A. flavus, A. parasiticus* and *A. nomius* produce the most carcinogenic mycotoxins, the aflatoxins [4], whereas as many as 10 species of *Aspergillus* produce ochratoxin A [5, 6, 7, 8]. Several *Penicillium* species are commonly involved in feed spoilage, and most of them produce more than 10 different toxic fungal metabolites (cyclopiazonic acid, patulin, citrinin, penicillic acid) [9, 10]. Although many *Fusarium* species exist in nature, only a small number infect cereal crops and feeds and produce mycotoxins (fumonisins, deoxynivalenol, zearalenone and trichothecenes) [11].

Swine are particularly sensitive to the effects of trichothecenes and fumonisins. Because of it, it's necessary to check the presence of moulds that are capable to generate these mycotoxins.

Based on these considerations, in this research was identified and characterized the mycobiota in swine feeds, commercialised in Portugal.

2. Material and methods

2.1 Sampling

A survey was carried out to evaluate the fungal contamination of 75 samples of swine feed. The samples were collected from factories of all country directly from the production line to the laboratory.

2.2 Mycological analysis

Mycological analysis was performed according official standard procedures (NP-3277-2, 2002; Food Microbiology – General rules for counting moulds and yeasts at 25ºC; Part 2: Reference method).

Ten grams of each sample were homogenized for 5 min in 90 ml (10^{-1} suspension) peptone water (Oxoid code CM 727; SR 78). Ten-fold dilutions were prepared till 10^{-3}. For enumeration and identification at genus level of moulds, 1ml of each dilution was spread into each of 4 plates (0.25 ml/plate) of Dichloran Rose Bengal Chlortetracycline Agar (DRBC) and incubated at 25ºC for 5 to 7 days. Each isolated mould colony was observed microscopically for morphological characterization and identification [12, 13].

3. Results and discussion

From the 75 mycological analyses carried through the swine feed samples only 2 granulated feed have not presented any type of fungal contamination (2.7%). The remains 73 samples had resulted positive (97.3%), corresponding to the 10 meal samples and the 63 granulated ones (Table 1).

Table 1- Number and frequency of positive and negative samples according to the commercial presentation.

	Meal		**Granulated**		**Total**	
	N	**(%)**	**N**	**(%)**	**N**	**(%)**
Positive	10	13.3	63	84.0	73	97.3
Negative	0	0.0	2	2.7	2	2.7
Total	10	13.3	65	86.7	75	100.0

Legend: N- sample number.

Since that the granulation process involves thermic treatments which result in decontamination of the processed material, it is expected to outcome a minor contamination in the granulated samples.

The mycological average content (M) was 6.6×10^2 cfu/g, oscillating between 2.7×10^1 cfu/g and 2.7×10^3 cfu/g. The most prevalent moulds belong to *Aspergillus* (84.9%) (M= 7.8×10^2 cfu/g), *Penicillium* (74%) (M= 1.0×10^3 cfu/g), *Cladosporium* (72.6%) (M= 1.5×10^2 cfu/g), *Mucor* (71.2%) (M= 2.2×10^2 cfu/g) and to *Fusarium* genera (61.6%) (M= 1.5×10^3 cfu/g). *Aspergillus flavus*, the unique specie of *Aspergillus* studied in these analyses, was detected in 52 samples (71.2%), presenting levels between 1.0×10^1 and 2.4×10^3 cfu/g. Yeasts were found in 69.9% of the positive samples, presenting a medium level of 2.7×10^3 yeasts/g. The less common moulds found were *Phoma* spp. (32.9%) (M= 2.7×10^1 cfu/g), *Rhizopus* spp. (35.6%) (M=5.3×10^1 cfu/g) and *Paecillomy* spp. (47.9%) (M=3.6×10^1 cfu/g) (Table 2; Figures 1 and 2).

Table 2- Mycological mean levels of different genera (cfu /g and Log_{10} cfu/g)

Fungi	Average (cfu/g)	Average (log_{10} cfu/g)
Yeasts	2.7×10^3	3.4
Asp. Flavus	6.9×10^2	2.8
Aspergillus spp (*).	7.8×10^2	2.9
Cladosporium spp.	1.5×10^2	2.2
Penicillium spp.	1.0×10^3	3.0
Mucor spp.	2.2×10^2	2.3
Fusarium spp.	1.5×10^3	3.2
Phoma spp.	2.7×10^1	1.4
Rhizopus spp.	5.3×10^1	1.7
Absidia spp.	1.1×10^2	2.0
Paeccillomy spp.	3.6×10^1	1.6
Mean Total Content	6.6×10^2	2.8

Legend: (*) with exception for *A. flavus*

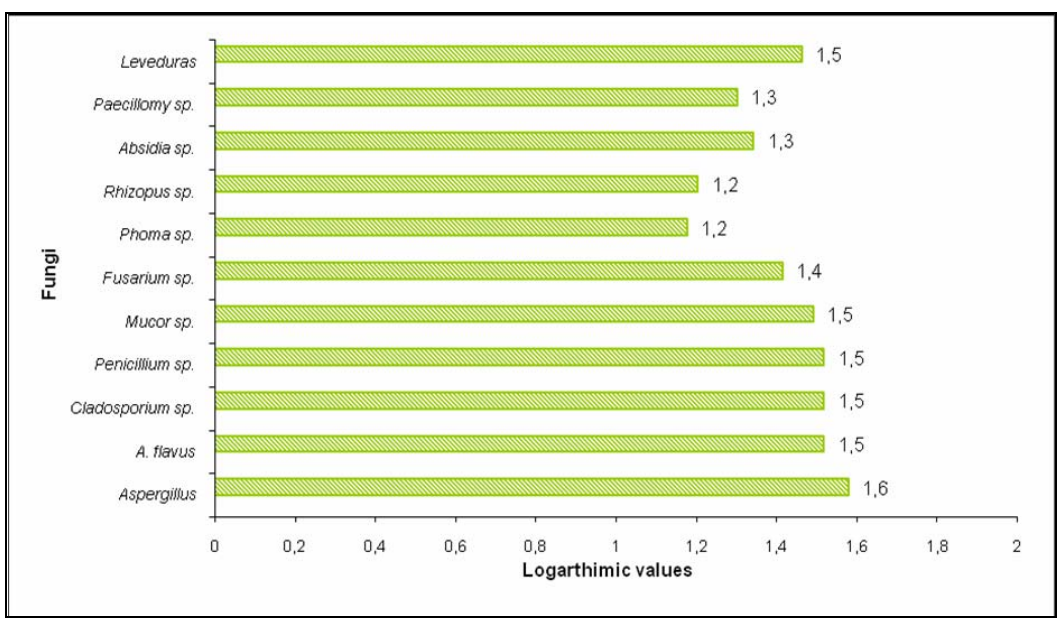

Figure 1 – Mean levels of different genera (Log_{10} cfu/g)

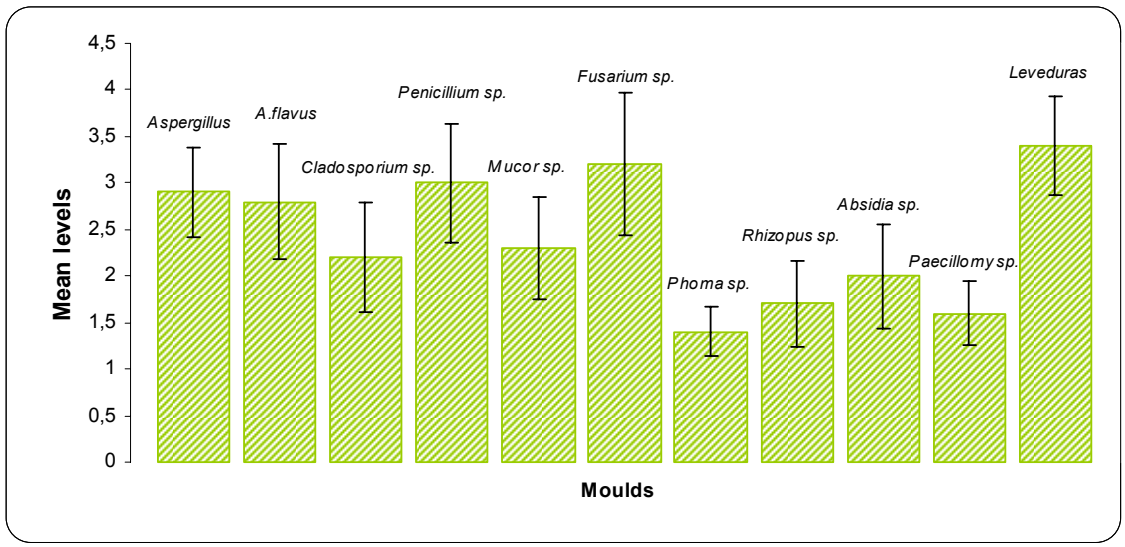

Figure 2- Mean levels (Log_{10} cfu/g) of different genera and respectively standard deviations.

From the analyzed samples, 68.5% presented satisfactorily reduced total mycological contents (TMC < 5.0 x 10^3 cfu /g) and are considered of good quality; 30.1% indicated not very high contents (5.0 x 10^3 < TMT < 5.0 x 10^4 cfu /g), but the average can motivate the adoption of extraordinary actions for the mycological control, to prevent fungal proliferation; 1.4% of the samples have a mediocre quality (5.0 x 10^4 < TMT < 7.0 x 10^5 cfu /g).

As it happens for raw materials, feedstuffs analysis based on total mycological contents is insufficient, being necessary to study the flora, able to identify the potentials toxic risks for the animals. Swine are particularly sensible to mycotoxins concretely, aflatoxins, zearalenone (ZEN) and deoxynivalenol (DON) [14, 15]. It is imperative to control the growth of moulds that are able to produce these mycotoxins, to prevent its synthesis.

In its majority, the feed samples analyzed in this study presented tolerable mycological quality. In 2001, Martins and Martins [16] had published that 86 of the 106 swine feed samples analyzed presented contaminations with a mean value 7.8 x 10^4 cfu/g. The most frequently moulds were *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor* genera, with mean levels more highly comparatively to the current results. After that, in Martins *et al*., 2005 [17] the examination of 261 swine feed samples resulted in contamination of 90.5%, with mean level 4.2 log_{10} cfu/g. *Aspergillus*, *Penicillium* and *Fusarium* genera were very frequently in these contaminated samples.

The illustrated results point to a positive perspective of the qualitative progress, concerning to fungal contamination levels in swine feed, having as reference studies carried through, in Portugal.

Acknowledgments The authors would like to acknowledge to Candida Camejo and Anabela Ramos for their collaboration in this work.

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Thermal inactivation of *Escherichia coli* and coliform in Oaxaca cheese curd during a simulated kneading process

I. Caro Canales[*,1], S. Soto Simental[1], I.L. Álvarez Contreras and J. Mateo[2]

[1] Food Science and Technology Research Centre, University of Hidalgo State, Avda. Universidad km 1. 43600, Tulancingo, Hidalgo, México
[2] Department of Food Hygiene and Technology, Campus Vegazana s/n, 24071 León, Spain

[*]Corresponding author: e-mail: dhticc@unileon.es, Phone: +5217751031577

The purpose of the present study was to asses the thermal inactivation of *E. coli* and coliform during the kneading step, in hot water, in the making process of Oaxaca cheese – a fresh pasta filata Mexican cheese. A three-strain cocktail of *E. coli* isolated from industrial Oaxaca cheeses was used for the assay. Three batches of pasteurised and, then, inoculated milk were processed into Oaxaca cheese following a traditional open-vat process until before the kneading step. At this point, the process was halted and a simulated kneading step at 55 ºC for 15 min was carried out. Counts of *E. coli* and coliform were performed on the curd after 0, 5, 10 and 15 min of the begining of the sumulated kneading. The data of these counts were analysed using linear regression, and the correspondent $D_{55ºC}$ values were calculated. The heating taking place during the kneading can reduce considerably the population of *E. coli* and coliform of the curd. However, it did not eliminate entirely the risk of foodborne illness in Oaxaca cheese.

Keywords microbial heat resistance; pasta filata cheeses; Mexican cheeses; *E. coli*; kneading

1. Introduction

Coliform and *Escherichia coli* have been commonly used as indicators of improper sanitation of food and water supplies. What is more, certain *E. coli* strains causing diarrhoeal illness are considered as emerging foodborne pathogens [1,2]. Cattle have been recognised as a principal reservoir of pathogenic *E. coli*, and the possibility of transmission through consumption of raw milk and raw-milk dairy products has been repeatedly documented [1,3]. In addition, contaminated pasteurised milk and the resulting diary products are also important sources of foodborne *E. coli* infections.

Some *E. coli* strains can grow at temperatures as low as 7ºC and as high as 46ºC, and the minimum pH at which growth occurs is approximately 4.0, under optimal temperature and water activity contitions, i.e. 25-37ºC and 0.975-0.985, respectively [4,5]. Regarding pH, in dairy products, it has been found that this microorganism can survive at around 5.0-5.4 [6,7,8]. Thermal sensitivity of *E. coli* O157:H7 has been studied in different matrices (foods and laboratory media) and at several temperatures [9,10,11]. The inactivation of infectious non-spore-forming pathogens using a heat treatment is a critical control point in food processing in order to eliminate the risk of food-poisoning outbreaks [12]. In a traditional approach it is assumed that microorganism thermal inactivation can be described by first order kinetics.

Oaxaca cheese is one of the most popular Mexican cheeses, which is widely used in Mexican dishes, usually melted. Oaxaca cheese presents similarities with low-moisture Mozzarella regarding cheese making process, physico-chemical and functional characteristics. Oaxaca cheese is a pasta filata cheese and, therefore, a characteristic step in its making process is the kneading of the curd, which is carried out into hot water, at 50-60 ºC, at the moment when curd pH reaches approximately 5.3. After that, long and thin straps of cheese are obtained by stretching, which are cooled in chilled water, removed, salted, cut into segments and, finally, these segments are coiled into the shape of balls. The mean pH value of Oaxaca cheese showed to be 5.1 and the water activity 0.991, with a moisture content of 51.5% [13]. In spite of being a fresh cheese, and that cheese standards require fresh cheeses to be made from pasteurised milk, Oaxaca cheese is normally produced from raw milk when made in small typical dairy plants. In this sense, in Mexican cheese sector, it is questioned to what measure the *E. coli* and coliform population is reduced during the hot-water-kneading step in raw milk Oaxaca cheese production process. Thus, the purpose of the present study was to asses the thermal inactivation of *E. coli* and coliform during that kneading step.

2. Materials and Methods

2.1 E. coli/Coliform counts in Oaxaca cheese and strain isolation

Three samples of raw milk Oaxaca cheese, weighting between 250 and 500 g each, were taken at three different industries in Hidalgo State (Mexico). Cheese samples were transported to the laboratory under refrigeration conditions.

From each sample, a 10-g aliquot was aseptically obtained by means of radial cuts [14]. and homogenized in 90 mL of buffered peptone water (CM 509, Oxoid Ltd., Basingstoke, U.K.), according to FIL (1996) [15] with a Stomacher 400 circulator (Seward, London U.K) during 2 min. Duplicated serial dilutions were made in buffered peptone water and, then, 1 mL of each dilution was plated on 3M Petrifilm *E. coli*/Coliform (Laboratories 3M Santé, Cergy Pontois Cedex, France). Afterwards, plates were incubated at 37ºC during 24-48 h, according to the AOAC official method numbers 998.08 and 991.014 [16], and typical colonies were counted – typical colonies for *E. coli* and coliform (other than *E. coli*) shows blue to red-blue colour and red colour, respectively, and both types of colonies are associated with an entrapped gas. Next, a total of 12 typical colonies for *E. coli* were isolated – four from each sampled cheese. Strains isolated were grew in BHI medium at 37ºC during 24 h and maintained at -20ºC in a 50%-glycerol solution.

2.2 Biochemical confirmation of strains and preparation of a cocktail

The strains isolated were confirmed as E. coli by the API 20E biochemical test strip using an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods (bioMériux, Lyon, France). Amongst those isolates, 3 strains which were confirmed to be E. coli were chosen to to obtain the three-strain cocktail utilized for the thermal resistance assay.

2.3 Thermal resistance assay during the simulated kneading process

Three batches of pasteurised milk, inoculated with about 10^4 ufc/mL of the *E. coli* cocktail (previously grown in BHI), were processed into Oaxaca cheese following a traditional open-vat process under standardised conditions until before the kneading step. At this point, the process was halted (curd pH was of 5.3) and a simulated kneading step at 55 ºC for 5 to 15 min was carried out – simulating the operational conditions used by the producers. For each batch, the curd was milled under aseptic conditions obtaining small curd particles (about 1 mm sized). Without delay, three portions of 20 g of curd particles were each loosely wrapped with sterile gauze and introduced in a 1-L-flasks full of water at 55 ºC, which in turn was submerged in water at this temperature. The curd samples were removed from the respective flasks at 5, 10 and 15 min. Counts of *E. coli* and coliform were performed on the curd immediately after milling or the removing from the flasks at the times indicated before, using 3M Petrifilm *E. coli*/Coliform plates. The data of these counts were analysed using linear regression analysis and the correspondent $D_{55ºC}$ values were calculated.

3. Results and Discussion

The numbers of *E. coli* and coliform observed in raw milk Oaxaca cheese are shown in Table 1. The high counts found in the analysis for both groups of microorganisms indicate poor hygienic conditions and imply a health risk.

Figures 1 and 2 illustrate the heat survivor curves of *E. coli* and coliforms (other than *E. coli*) in the curd of Oaxaca cheese during conventional kneading time period and temperature (10-15 min at 55 ºC, respectively). The survivor curves demonstrate a linear decline in the Log number of viable cells as a function of heating time. In addition, table 1 shows the $D_{55ºC}$ value and the coefficient of determination (R^2) obtained with the linear regression analysis for each batch, as well as the calculated number of logarithm reductions during 15 min of kneading.

Table 1 Coliform and *E. coli* and counts (log cfu g^{-1}) in raw milk Oaxaca cheese

	E. coli	**Coliform***
Media±SD (n=3)	2.97±1.19	4.03±0.87

* Coliform other than *E. coli*

The curd before kneading had 5.0±0.7 Log ufc g^{-1} *E.coli*, which was a value higher (roughly 1 logarithmic unit) than the concentration of *E. coli* in the inoculated milk. This fact could be due to the concentration of *E. coli* during the cheese making process. Additionally, the curd contained 5.1±0.2 Log ufc g^{-1} of other coliform different from *E. coli*. The presence of these coliform in the curd could be explained by post-pasteurisation contamination of milk and/or curd in the vat, which was an open vat. According to Robinson and Tamime (2002) [17], the microbiological quality of cheese is influenced by equipment and environmental hygiene during manufacture and handling.

During a conventional kneading time period of 15 min at 55 ºC a 4.7±0.6 logarithmic decrease in *E. coli* concentration was calculated, being 3.5±0.2 for coliform concentration. The resulting mean $D_{55ºC}$ values (time required at 55 ºC for 1-log unit reduction in counts) were 3.1±0.5 min for *E. coli* and 4.3±0.4 min for coliform other than *E. coli* (Table 1).

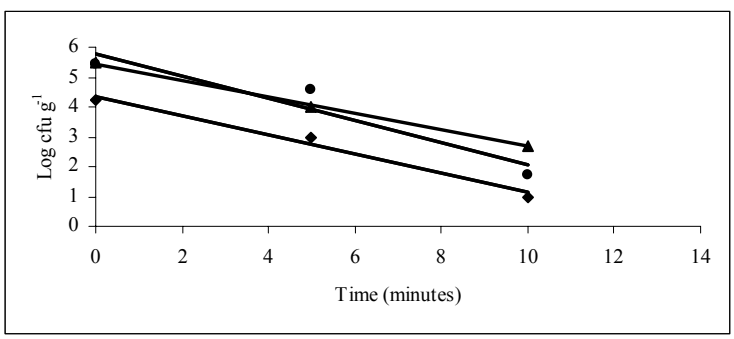

Fig. 1 Survivor curve of *E. coli* in the curd of Oaxaca cheese mantained at 55ºC. Data points were from the average of three measurements.

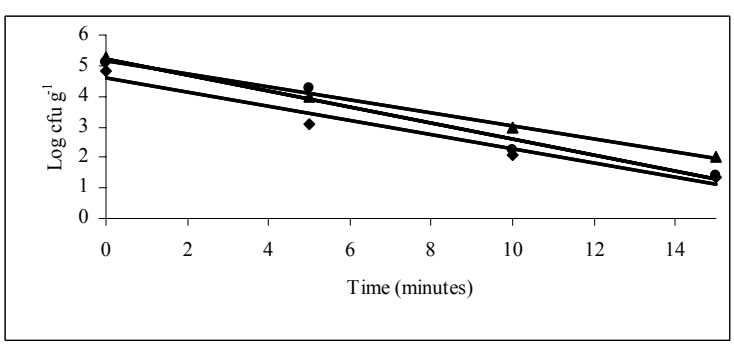

Fig. 2 Survivor curve of coliform (other than *E. coli*) in the curd of Oaxaca cheese at 55ºC. Data points were from the average of three measurements.

D55ºC values for *E. coli* in the curd of Oaxaca cheese were between the values of 1.9 and 5.2 found by Li-Wuan-Po et al. (2002) [18] and Jin et al. (2008) [19] in simulated apple cider and liquid egg, respectively. However, those values were lower than the 21.1 min reported for *E. coli* O157:H7 strains in beef and the 9 to 38 min reported for *E. coli* in meat in general [20,21]. Discrepancies can be partially explained by compositional differences in the correspondent food matrix, since the environment in which cells are immersed during the heat treatment has a significant effect on their heat resistance. In general, it is expected a decrease in the heat resistance of bacteria with decreasing the pH. However, Blackburn et al. (1997) [11], when studying the effect of pH on heat resistance of *E. coli* O157:H7 in tryptone soya broth with different concentrations of common salt, found that the pH range to which this microorganism showed the higher resistance was between 5.2 to 5.8. Oaxaca cheese curd pH is at the lower limit of that range, so that, following the model by Blackburn et al. (1997) [11], pH would not seem to affect the thermal resistance of *E. coli*.

Table 1 $D_{55°C}$ values of *E. coli* and coliforms in the curd of Oaxaca cheese during the heat treatment of the sumulated kneading, R^2 and log unit reduction.

	$D_{55°C}$ values (min)	R^2	Log unit reduction (Log ufc g^{-1})
E. coli *(test number)*			
1	2.70	0.906	5.3
2	3.62	1.000	4.1
3	3.11	0.981	4.7
Media±SD	3.10±0.46		4.70±0.60
Coliform other than *E. coli* *(test number)*			
1	4.3	0.962	3.5
2	3.8	0.970	3.7
3	4.6	0.995	3.2
Media±SD	4.30±0.42		3.5±0.23

R^2: Coefficient of determination of the linear regression
Log unit reduction: Number of log unit reduction in the curd after 15 min of heat treatment R^2

By means of this study has been demonstrated that the heating taking place during the kneading step in Oaxaca cheese making process can reduce considerably the counts of *E. coli* and coliform of the curd. However the observed reductions suggest that kneading did not eliminate entirely the risk of foodborne illness in Oaxaca cheese due to a previous pathogenic *E. coli* contamination of the curd.

Acknowledgements The support by the Mexican Governmental PROMEP-SEP programme is gratefully acknowledged.

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Using food industry wastes for producing valuable materials: Skin of eggplant for wool dyeing

M. Parvinzadeh[*,1] and **M. Haji Ashrafi**[2]

[1]Young Researchers Club, Islamic Azad University, Shahre Rey Branch, Tehran, Iran
[2] Faculty of Postgraduate, Islamic Azad University, South Tehran Branch, Tehran, Iran

[*] Corresponding author: e-mail: mparvinzadeh@gmail.com, Phone: +98 9123137115

Today, natural colorants are emerging globally due to the fact that are safer and environmentally-friendly. Natural dyes have been employed in dyeing Persian carpet piles for many years. The beautiful colour shades made from endemic plants together with the unique patterns contribute to the world fame of Persian carpet.
Eggplant (Solanum melongena) is a member of the Solanaceae, a large, diverse plant family containing 18 different domesticated species. Anthocyanin pigment in the skin gives the fruit its familiar dark purple colour. In this study, the skin of eggplant was powdered and it was used for dyeing wool yarns. The Iranian wool yarn was first scoured with nonionic detergent, mordanted using some metal salts including Fe(II), Sn(II), Cu(II), Cr(VI) and Al(III). It was then dyed with 50% owf skin of eggplant. The colorimetric properties of the dyed yarns were evaluated with reflectance spectrophotometer. The wash and light fastness of the samples were measured according to ISO 105-CO5 and Daylight ISO 105-BO1. Results showed that skin of eggplant is a susceptible food waste for dyeing of protein fibers as rug piles.

Keywords Wool; Dyeing; Anthocyanin

1. Introduction

For several centuries humans admired the beautiful natural colors of plants and minerals. The natural dyes have been known and used for thousands of years for painting of body and making foods for ancient humans [1]. The greatest use of natural dyes occurred when the art of weaving developed [2]. The rug weavers also used these dyes for dyeing of rug piles but production and development of synthetic dyes limited using of natural dyes [3]. Synthetic dyes offered greater variety and stability of color as compared to natural dyes and the dyers preferred to use synthetic dyes more and more [4]. But today dyeing processes based on natural sources have gained importance in view of stringent environmental and industrial safety conditions which innovative ideas in dyeing industry are being tried to cater to such needs [5,6). Natural dyes processed for the market do not undergo any chemical operations. Those operations involved are purely physical, such as grinding, drying and water extractions. None of these operations create any great environment problems [7]. Special color effects are also achieved in fibers dyed with natural dyes with good washing properties. The combination of natural sources with new processes could improve to expand into value added consumer goods [8]. In an age of consumer activism and increasing international trade of rugs and carpets, fastness tests and procedures assume more and more importance. Generally accepted standards provide a common basis for the evaluation of quality of natural dyed piles [9].

Many studies have continued into natural dyes covering such areas as variation in the quantity of dyes in plants or other sources, combination of dyes, properties of natural dyes, effects of mordant and auxiliaries on different properties of dyed samples, improvement of production of natural dyes and discovering of other natural sources. The fastness properties of natural yellow dyes including henna (lawsonia inermis), dolu (rheum emodi), kamala (mallotus phillipinensis), onion (allium cepa) and turmeric (curcuma tinctoria) with different mordants have been studied by M. Gulrajany et al (10,11,12]. They concluded that the presence of the mordant does not affect the fastness characteristics of henna, kamala and turmeric but onion and dolu are capable of forming stable complexes with metal salts and the application of mordant improves the fastness properties of these two dyes. Syed Ishrat Ali also suggested that in dyeing with natural dyes, mordants combined chemically with a soluble dye to form a complex and the fastness properties depend on the formation of complex inside the textile fiber [13].

The eggplant, a member of the deadly nightshade family, is a vegetable of dubious origin. Some say it originated in China some 4000 years ago, then was introduced into the Mideast by Arab traders in the 8th century. Others say it originated and was domesticated in India, then brought home by members of Arab armies in the 7th century. Others yet say it has been grown and eaten as a vegetable in Iran since 1500 BC [14, 15].
Americans are the only ones who call it "eggplant." They're "aubergines" to the French and British--from the Arabic bathinjan, which itself was Arabized from the Persian "badnjan"; "berenjena" to the Spanish and

"berinjela" to the Portuguese, for the same reason; "melanzane" and "melitzane" to the Italians and Greeks, deriving from the 16th century scientific classification of mala insana, or "mad apple"[16-18].

Fruit and plant color is affected during eggplant domestication. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom which present at skin of eggplant. They are also responsible for most of the red, purple, and blue colours exhibited
by flowers, fruits and other plant tissues and have found applications in the food industry as natural colorants. Eggplant cultivars display a diversity of colors due to the presence or absence of anthocyanin and chlorophyll in fruit tissue and the light sensitivity of pigment synthesis. Cold-stressed plants often exhibit abnormally high anthocyanin expression. The pigment is an antioxidant with anti-cancer properties [19].

2. Experimental

2.1. Materials

The wool was Iranian yarn of 430/2 tex and 144 twists/m. The nonionic detergent was used for the scouring of wool yarns and it was obtained from Shirley Development Limited. Mordants including aluminium potassium sulfate, copper sulfate and ferric sulfate from Merck were used for pre-mordanting of wool yarns. Acetic acid 85% (Merck) was applied for mordanting and dyeing processes. Skin of Iranian eggplant was powdered and it was used for dyeing.

2.2. Procedures

Scouring: The yarns were scoured in 5% nonionic detergent. The L:G (liquor to good ratio) of the scouring bath was kept at 50:1 for 25 min at 75ºC.

Mordanting: The scoured yarns were divided into ten parts. One part was retained untreated for reference and the others were each mordanted with aluminium potassium sulfate, copper sulfate, potassium bichromate, stannous chloride and ferric sulfate (5, 10 and 15% w/w). The L:G of mordanting was 50:1. Acetic acid was used in the mordanting bath for adjusting the pH 5. The process was started at 40ºC and then was gradually raised to the boiling point during 20 min. Finally the mordanting bath was boiled for 1hour.

Dyeing: Powder of skin of eggplant was poured (skin of eggplant: water = 10: 1) into water and left for 24 h. The pH of the dyebath was kept at 5 by adding 1.5% owf acetic acid. The skin of eggplant concentration was 50% owf and L: G was kept at 50: 1. The dyeing started at 40°C and was raised to 85°C over 20 min. Finally, dyeing was carried out at this temperature for 1 h.

Reflectance measurement: CIELAB color coordinates of dyed samples (L*, a* and b*) were measured using a spectrophotometer COLOREYE 7000A from Gretagmacbeth integrated with an IBM computer. Color co-ordinates were calculated from the reflectance data for 10º observer and illuminant D65.

Determination of color fastness: The wash-fastness properties of the dyed yarns were measured according to ISO 105-C01. The color hue changes of the yarn and the degree of staining on the adjacent yarns were measured after drying.

For light-fastness measurements, the yarns were exposed to the daylight for 4 days according to the daylight ISO 105-B01, and the changes in the color (fading) were assessed by the blue scale.

3. Results and Discussion

3.1. Color measurement

The results of color measurement of mordanted/dyed samples are shown in Table 1. The amount of L* (lightness) was decreased for all the samples treated with 1% mordants and it continued to decrease with increase in salts concentration to 3% and 5% in mordanting bath. Pre-treatment of fibers with different salts caused to increase the anthocyanin absorption into the wool fibers and the color shade of wool fibers was changed for different salts. Copper sulfate decreased the a* values (redness) comparing to other mordanted samples and hue was changed from yellow-red region to green-red region.

Table 1 Amounts of L*, a* and b* of mordanted/dyed wool yarns

Sample	Mordant %	L*	a*	b*
Non-mordanted	**-**	**70.06**	**5.27**	**37.94**
Aluminium potassium sulfate	**5**	**63.34**	**5.77**	**36.34**
	10	**61.96**	**6.31**	**33.98**
	15	**59.63**	**6.10**	**31.66**
Copper sulfate	**5**	**44.34**	**-0.73**	**28.32**
	10	**42.65**	**-0.90**	**26.02**
	15	**40.64**	**-0.91**	**25.55**
Potassium Bichromate	**5**	**56.96**	**3.47**	**37.53**
	10	**53.14**	**3.85**	**37.18**
	15	**52.02**	**4.02**	**37.74**
Ferric sulfate	**5**	**52.74**	**8.73**	**20.11**
	10	**52.08**	**8.33**	**20.53**
	15	**51.76**	**8.76**	**19.45**
Stannous chloride	**5**	**65.77**	**5.77**	**41.11**
	10	**65.36**	**6.31**	**41.74**
	15	**64.98**	**6.10**	**41.96**

3.2. Fastness properties

Table 2 shows the wash fastness for mordanted/dyed wool yarns. All the mordants improved the wash fastness and staining behavior on wool and cotton. Improve in wash fastness properties could be the result of making complex between metal salts and anthocyanin which has been confirmed for dyeing of wool with other kind of natural colorants.

The results of light fastness test are shown in Table 3. According to results, light fastness was improved for all the mordanted yarns. Stannous chloride shows the best light fastness properties on wool yarns comparing to other mordants. Mordants forms complex with dyes resulting light fastness improvement. Light fastness results allow the results obtained from wash fastness measurement.

Table 2. Results of Washing-Fastness tests for mordanted/dyed wool yarns

Sample	Mordant %	Wash fastness	Staining on wool	Staining on cotton
Non-mordanted	**-**	**4**	**4**	**4**
Aluminium potassium sulfate	**5**	**4-5**	**4-5**	**4-5**
	10	**4-5**	**4-5**	**4-5**
	15	**4-5**	**4-5**	**4-5**
Copper sulfate	**5**	**4-5**	**4-5**	**4-5**
	10	**4-5**	**4-5**	**4-5**
	15	**4-5**	**4-5**	**4-5**
Potassium bichromate	**5**	**4-5**	**4-5**	**4-5**
	10	**4-5**	**4-5**	**4-5**
	15	**4-5**	**4-5**	**4-5**
Ferric sulfate	**5**	**4-5**	**4-5**	**4-5**
	10	**4-5**	**4-5**	**4-5**
	15	**4-5**	**4-5**	**4-5**
Stannous chloride	**5**	**4-5**	**4-5**	**4-5**
	10	**4-5**	**4-5**	**4-5**
	15	**5**	**4-5**	**4-5**

Table 3. Results of light-Fastness tests of mordanted/dyed wool yarns

Sample	Mordant %	After 4 days
Non-mordanted	**-**	**4-5**
Aluminium potassium sulfate	**5**	**5**
	10	**5**
	15	**5**
Copper sulfate	**5**	**5-6**
	10	**6**
	15	**6**
Potassium bichromate	**5**	**6**
	10	**6**
	15	**6-7**
Ferric sulfate	**5**	**5**
	10	**5-6**
	15	**6**
Stannous chloride	**5**	**7**
	10	**7**
	15	**7**

4. Conclusion

The wool yarns were first mordanted with aluminium potassium sulfate, copper sulfate, potassium bichromate, stannous chloride and ferric sulfate, and then dyed with powder of skin of eggplant. Colorimetric and fastness properties of dyed yarns were measured. According to the results, the amount of L* was decreased for all the mordanted samples comparing to non-mordanted one. Copper sulfate decreased the a* values comparing to non-mordanted samples and hue was changed.

Mordants form complex with crocine resulting wash and light fastness improvement.

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Industrial Microbiology. Future Bioindustries

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Application of Biotechnology in Petroleum Industry - Microbial Enhanced Oil Recovery

Debanjan Sengupta[1,*]

[1] Department of Biotechnology, School of Biongineering, SRM University, Kattankulathur, Kancheepuram Dist. – 603203, Tamil Nadu, India

[*]Corresponding author: e-mail: debnjn_sengupta@yahoo.co.in

Biotechnology has gained popularity in the Petroleum Industry in recent times due to its environment friendly approach. Various jobs undertaken by the Petroleum Industry involves high cost, complex operational implementation and causes environmental hazards. Implementation of Biological systems in these kinds of jobs is a very good alternative.

Microbial Enhanced Oil Recovery (MEOR) is the usage of microorganisms to facilitate and increase oil production from an oil reservoir. This technique is applied after the primary and secondary techniques of oil recovery. It is estimated that in most of the oil reservoirs, more than 60% of the oil still remains in the well. Hence importance of this technique is validated. Also, it is a low cost process, and it uses the ability of microorganisms to produce a wide range of metabolites, which assist in oil mobilization by lowering oil viscosity and interfacial tensions, thereby, culminating into enhanced oil recovery.

Keywords Petroleum Industry; Microbial Enhanced Oil Recovery (MEOR).

1. Introduction

There are three phases in the extraction of oil from a well:

- Primary Phase
- Secondary Phase
- Tertiary Phase

Primary Phase involves the discovery of an oil field. Then, using natural stored energy, oil is moved out by the expansion of volatile components or by external pumping to assist the natural drive. The natural forces that are involved are:

1. Expanding force of trapped natural gas.
2. Gravitational force (in case of steeply inclined reservoirs).
3. Expulsive force due to compaction of poorly consolidated reservoir rocks.

Slowly, the natural energy gets depleted and the production declines.

In the **Secondary Phase** of extraction of oil, water is injected into the well. Then, the oil and water mixture is extracted. But after sometime the oil to water production reaches the economic limit of operation. Net profit diminishes because the production of oil becomes too narrow. This process is then stopped.

Then the **Tertiary Phase** of oil recovery or *Enhanced Oil Recovery (EOR)* techniques are applied. It involves the use of physical, chemical and thermal energy from an externally supplied source. Few of the major EOR techniques are — Steam Injection, In-situ Combustion, Surfactant-Polymer Injection, CO_2 Injection, Microbial Enhanced Oil Recovery (MEOR), etc.

It is estimated that combined oil production by primary and secondary methods is less than 40% of the original oil in place. Thus, the score of EOR is better than any of other conventional methods.

2. Theory

MEOR is one of the most promising biotechnological techniques that can be successfully applied in the Petroleum Industry worldwide. It involves the usage of microorganisms to facilitate, increase or extend oil production from an oil reservoir[5].

2.1 Advantages of MEOR technique

- It is a cost effective process.
- Microbes produce a wide range of metabolites that actually enhances oil production.

- Microbes are self duplicating. Hence, a little input of microorganisms will result in its high proliferation and thus, high production of metabolites.
- It is operationally simple and does not require any deployment of any rig.

2.2 Method of enhancement of oil recovery

The recovery of oil is enhanced by the help of the secondary metabolites that are produced by the bacteria[4].

Table 1: Effect of metabolites on the crude oil inside the reservoirs.

METABOLITES/BIPRODUCTS	EFFECT
Acids	•Modification of Reservoir Rocks. •Improvement of porosity and permeability. •Reaction with calcareous rocks and production of CO_2.
Gases (CO_2, CH_4)	•Reservoir repressurization. •Oil swelling, Viscosity reduction. •Increase in permeability.
Solvents	•Dissolving the oil.
Surface Active Agents	•Lowering of interfacial tension. •Emulsifications.
Polymers	•Mobility control. •Selective or non-selective plugging.
Biomass	•Degradation and alteration of oil. •Reduction of oil viscosity.

3. Methodology

3.1 Properties of desired microbial culture

- It should be able to sustain the reservoir conditions, such as, high temperature, high pressure, high hydrocarbon content, high salt concentrations, anaerobic conditions etc.
- It should be able to proliferate normally in the medium.
- They should produce the desired metabolites.
- They should be non-pathogenic.

3.2 Selection of the microbial culture

In India, MEOR is applied by Oil and Natural Gas Corporation in some of its oil fields in the state of Assam. Initially, tests were done to identify the microbial species that satisfy the above stated conditions. It was found that several species of the bacteria of *Clostridium Sp.* and the Sulphur Reducing bacteria (SRB) are suitable for the MEOR techniques in the reservoir conditions. But SRB has got several adverse effects, such as, production of harmful H_2S gas, and it is also involved in biocorrosion of iron and steel storage tanks and pipelines.

The bacterium of *Clostridium Sp.* is used in this technique. It is isolated from either formation water or from other sources like crude oil, contaminated soil samples, celler pits, etc.

It is isolated and grown in molasses based selective media containing carbon, nitrogen, phosphorus, oxygen and other essential salts (K_2HPO_4, KH_2PO_4, $FeSO_4$, $MgSO_4$, zinc, manganese, cobalt, copper etc.). A colour of the culture medium from black to brown indicates good bacterial growth. The pH of the medium is maintained at slightly basic conditions.

The Indian oil major, Oil and Natural Gas Corporation, has developed and patented three species of thermophilic, halophilic, barophilic and anaerobic microbial consortium (*C. thermoanaerobacterium*, *C. thermotoga*, *C. thermococci*) that is active upto 90°C. Worldwide, the technology is applicable upto 75°C.

3.3 Optimization of the culture

a) Standardization of Molasses Concentration:
The molasses concentration of the specified medium was varied to observe its effect on the metabolite production, bacterial count, etc. The concentration at which these factors are the highest is taken for the culture.

b) Selection of Buffers:
Different buffers (HEPES, MOPS, Sodium acetate etc) are used to see their effect on gas production ability of the bacteria. The buffer which induces maximum gas production is selected.

3.4 Acclimatization of the bacteria to the reservoir conditions

The well temperature is higher than the surface temperature. In a well as deep as 1500m to 2000m, the well temperature is around 70°C. The bacteria that was isolated and optimized in the laboratory, has to be acclimatized to sustain at higher temperature, that is, it has to be made thermophilic.

The temperature of the culture was raised by 2°C per week, till it reached 50°C. Then it was raised by 1°C per week, till it reached 75°C. The growth was observed by measuring

- The produced gas
- pH
- Sugar Content
- Bacterial Count (by serial dilution method)

The isolation technique of the microbial consortium, stable at 90°C is not discussed here.

3.5 Preparation of bulk culture

Bulk culture is produced in a bioreactor by inoculating designed nutrient medium with fixed amount of active culture. It is prepared under anaerobic conditions and at a bottom-hole temperature of well under test. The fully grown active culture is then transferred from the bioreactors to specially made insulated containers by maintaining aseptic and anaerobic conditions and then transported to the site of application.

3.6 Practical treatment sequence

Practical treatment sequence of the cultures in the wells involves the following steps:

I. Well Selection – It involves selection of an appropriate well that is assumed to contain large amount of trapped oil.

II. Job Designing – Planning the steps to execute the technique. It mainly involves the calculation of the amount of the bulk culture required and the nutrient media that is to be injected into the well.

III. Job Execution – It mainly involves the injection of the culture, water, molasses and the nutrient media into the well (HUFF). The well is then closed for six to eight weeks (SHUT IN). Around 120m^3 to 200 m^3 of the culture is to be injected for a well of 6-8 m radius. The well is then opened for the extraction of the well (PUFF).

IV. Monitoring – Amount of metabolites produced, gas evolved, oil extracted etc. are monitored regularly in this step.

3.7 Mechanism of action of bacteria

- Reduction of the interfacial tension.
- Swelling of oil and reduction of its viscosity.
- Increase of pore pressure and physical displacement of oil from rock surface.
- Increase of porosity and permeability.

It is not the bacteria itself, but, the metabolites produced by it perform the above job[4].

3.8 Extraction of oil

The oil now has reduced viscosity, interfacial tension and is more permeable. Therefore, it is then easily extracted from the reservoirs by the Sucker Rod Pumps (mechanism of which is like a normal hand pump, gas lift techniques (pumping of gas into the well and lifting up the oil), or other prevalent techniques.

4. Result

This technique is applied in various dormant oil fields in Assam in India by ONGC. The exact results, indicating the growth of the microorganisms in the well, amount of increase in oil extraction in the wells and other geochemical and geomicrobial properties are kept as company secrets and are not discussed openly. But, the outputs were very encouraging.

a) During monitoring process, crude oil samples were taken out and were analyzed in the lab for bacterial enumeration by serial dilution method followed by counting of colony forming units. It indicated a rise in the bacterial count thereby suggesting a healthy growth and reproduction of the bacteria.
b) The oil production increased considerably, though the exact increase is not revealed.

Hence, the MEOR technique is validated.

5. Discussion

I. There are 1147.7 thousand million barrels of proved oil reserves currently present in the world[5]. Out of this maximum 50% of it can be extracted by the primary and the secondary methods of oil recovery. Therefore, more than 600 thousand million barrels of oil are left to be extracted by the Enhanced oil recovery methods.
II. Due to the ever rising price of crude oil in the international market, it is becoming essential, day by day, to get the extraction cost reduced. Various Chemical Enhanced Oil Recovery techniques (like, polymer injection, CO_2 injection, in situ combustion etc.), that are used, are highly expensive. Compared to that, cost of MEOR is negligible, as all the raw materials required are of nominal cost and there is no need of much labour or complex machinery. Therefore, the extraction cost can be reduced considerably.
III. Naturally fractured oil reservoirs represent over 20% of the world's oil reserves. However, relatively little success has been achieved in increasing oil production from these complex reservoirs. Recent studies have shown that MEOR can be very successfully applied in oil recovery in such kind of structurally complex wells[1].

Acknowledgement I thank the researchers from Institute of Biotechnology and Geotectonic Studies, Oil and Natural Gas Corporation (ONGC), Jorhat, Assam, India for providing me an insight of MEOR.

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Development of biotechnological processes using glycerol from biodiesel production

Juan Daniel Rivaldi [1,2], **Boutros Foaud Sarrouh** [1], and **Silvio Silvério da Silva**[*,1,3]

[1] Escola de Engenharia de Lorena, Universidade de São Paulo, CEP: 12610-810, Lorena-SP, Brazil
[2] Facultad de Ciencias Químicas, Universidad Nacional de Asunción, CP: 1055 San Lorenzo, Paraguay
[3] Faculdades Integradas Teresa D'Avila, CEP: 12.606-580, Lorena-SP, Brazil

[*]Corresponding author: e-mail: silvio@debiq.eel.usp.br, Phone: +55 12 3159-3165

Given the increasing demand for reducing environmental pollution by using clean energy, there is an urgent need to investigate new and more efficient alternatives for renewable resources use and clean energy production. Although biofuels such as, biodiesel represents a secure, renewable and environmentally safe alternative to fossil fuels. Its production is increasing considerably, and as a consequence, the amount of crude glycerol (main by-product) generated is growing exponentially. In order to solve future environmental problems of glycerol accumulation and to turn the biodiesel production economically viable, implementation of biotechnological strategies that use glycerol as the only carbon source to co-produce higher value products along with biofuels has been proposed as a solution to this problem. In this work it will be presented a well documented argument on the metabolic mechanism of different microorganism for glycerol assimilation. As well as description of different biotechnological processes using glycerol as substrate for bioconversion into different industrial bioproducts in Brazil.

Keywords biodiesel; glycerol; assimilation; fermentation; bioproduct

1. Introduction

Environmental concerns for the reduction of the global and local pollution stimulate the biofuels market in the world. The global economy keeps growing and the demand of clean energy and renewable resources increase uninterruptedly [1]. Biofuels as biodiesel are highly valuated in development countries, because its production creates additional jobs and could make possible the desirable social development [2]. Biodiesel is produced by transesterification of triacylglycerol with alcohol (methanol or ethanol) generating fatty acids methyl or ethyl esters, and glycerol as principal co-product. Glycerol ($HOCH_2$-$CHOH$-CH_2OH) or 1,2,3-propanetriol is also obtained as a by-product from the manufacture of soaps and in less proportion from microbial synthesis. For every 9 kg of biodiesel, approximately 1 kg of crude glycerol is generated [3].Today, many countries made compulsory the addition of biodiesel to petroleum-based fuel. Brazil is an agro-industrial country that presents several advantages for the production and commercialization of this biofuel, due to the abundant raw-materials and continuous growth of vegetable oil and ethanol industries [4]. In 2013, the amount of biodiesel to be added most achieved 5 % (v/v) of common diesel in Brazil [5]. Increasing the volume of biodiesel, the co-produced glycerol also will increase from 83 to almost 366 millions of litres per year [6]. Consequently, the problems related to environment control will also increase. The low cost of crude glycerol in the market (0.2 – 0.4 $/kg) is attributable, not only to the high volume available, but also to the impurities contained as water, salts, esters, alcohol and residual oil [7]. Process for glycerol purification mainly includes filtration, vacuum distillation, bleaching, and ion exchange for K^+ and Na^+ removing. Typically, glycerol valorisation includes direct chemical transformation to useful compounds as feedstock in food, cosmetic, pharmaceutical and chemical industries. However, the purification treatments are too expensive for small and medium size biodiesel producers, due to this fact; more and more quantity of effluents containing glycerol could be discharged in the environment without any treatment. The microbial conversion of glycerol to different products as single cell protein, 1,3-propanediol, citric acid, succinic acid, dihydroxyacetone, polyhydroxyalkonates and more recently hydrogen, constitutes an alternative for the glycerol valorization and for a feasible biodiesel production [8, 9, 10]. This review presents detailed and well documented arguments of microbial mechanisms for glycerol assimilation, as well as, recent biotechnological processes used for raw glycerol bioconversion into different high-added value bioproducts in Brazil.

2. Characteristics of raw glycerol

Glycerol from biodiesel generally is a viscous liquid with range in colour from lightly yellow to dark brown. It contains a mixture of varying amount of soap, alcohol (methanol or ethanol), mono-acylglycerol, di-

acylglycerol, glycerol oligomers, polymers and water. The amount of glycerol in the mixture mainly depends of the feedstock source. It typically contents 40 to 70 % (w/w) of glycerol, with a majority of the rest of it being soap formed during the reaction of free fatty acids with excess of basic catalyst. Independently of the method utilized for biodiesel production, concentrated acid (H_2SO_4, HCl, or H_3PO_4) is added to the raw glycerol in order to remove soap [7]. After that, the glycerol can be recovered by a simple decantation with a concentration higher than 80 % (w/w). This glycerol contains salt in concentrations of 10 to15 % w/w as result of neutralization of excess of acid [7, 11]. The presence of such high salt concentration causes growth inhibition of many microorganisms or limits glycerol assimilation in fermentative process. The physical, chemical and nutritional characteristics of crude glycerol depend on the types of crude oil (animal fat, vegetable oil) and of catalysts (NaOH, KOH) utilized in biodiesel production [7, 11, 12]. Analytical tests of raw glycerol (derived from biodiesel using different feed-stocks) showed that it already presents elements such as, phosphorous, sulphur, magnesium, calcium, nitrogen and sodium that can be assimilated by microorganisms for their growth during fermentation [12].

3. Microorganism mechanisms for glycerol bioconversion

Glycerol, a highly reduced carbon source, plays an important role as substrate and energy source for numerous species of microorganisms. Glycerol is precursor of important cell compounds and regulator of different metabolic pathways, redox potential and phosphate recycling in cell [13]. Nowadays, several studies are being conducted for glycerol utilization under aerobic or anaerobic conditions [8, 9, 10]. The researches are mainly focused on bacterial fermentation to obtain intermediaries compounds for polymer production, resins and fuel additives. Yeast and bacteria can uptake glycerol into the cell by passive or active transport [14, 15,]. The passive transport includes simple diffusion (non-specific permeation) and facilitated diffusion (protein mediated transport). Experiments with *Saccharomyces cerevisiae, Candida utilis* and others yeast strains showed that membrane permeability for glycerol molecules can take place either by simple diffusion or by facilitated diffusion [15, 16]. Gancedo et al. (1968) working with *Candida utilis* and *Saccharomyces cerevisiae* observed that, *C. utilis* can grow on glycerol as easily as on glucose because of its high cell membrane permeability thus allowing a simple diffusion of the small glycerol molecules into the cell, on the other hand, *Saccharomyces cerevisiae* showed relative low permeability when compared with *C. utilis*. Further experiments demonstrated that, glycerol can permeate through *Saccharomyces cerevisiae* plasma membrane by means of transport proteins known as FPS1 and YFL054c [17]. Recently, active transport, symporter H^+- glycerol and symporter Na^+-glycerol transports (coupled to metabolic energy) were confirmed for *Saccharomyces cerevisiae* [16, 17] and E*scherichia coli*, mainly, in conditions of osmotic stress [17]. However, genes that encode these systems are still unknown [15, 17,].

After glycerol transport into the microbial cell by the mechanisms cited above, it can be metabolized aerobically by two parallel pathways (Figure 1). One of the routes, probably, the main pathway for glycerol oxidation in bacteria and yeast species, consists in glycerol phosphorylation by the enzyme glycerol kinase to produce glycerol-3-phosphate, which is then converted to dihydroxyacetone phosphate by the mitochondrial enzyme glycerol phospho-ubiquinone oxidoreductase (FAD-dependent) [14, 15]. Several reports inform that, the genes controlling the synthesis of glycerol kinase and phospho-ubiquinone oxidoreductase are GUT1 and GUT2, respectively [15, 17]. The expression of these enzymes is repressed during cell growth on fermentable substrates like glucose, but unregulated when glycerol or ethanol are used as main carbon source [17].

Another possible route, known as the dihydroxyacetone pathway (route observed with *Schizosaccharomyces pombe* under osmotic stress), includes the oxidation of glycerol by the enzyme glycerol dehydrogenase to form dihydroxyacetone, which is subsequently phosphorylated to dihydroxyacetone phosphate by the dihydroxyacetone kinase (ATP-dependent) [14]. The dihydroxyacetone phosphate is an important intermediary compound for gluconeogenesis and also for obtaining several bioproducts through oxidative pathways, such as citric acid, succinic acid, acetic acid and ethanol. In addition, dihydroxyacetone can be oxidized by the pentose-phosphate-cycle as verified with *Acetobacter suboxydans* [18].

In species of microorganisms as *Yarrowia* sp., *Klebsiella pneumoniae*, *Clostridium pasteurianum*, *Citrobacter freundii*, *Clostridium butyricum*, *Enterobacter agglomerans*, *Lactobacillus brevis*, *Lactobacillus buchneri* and *Bacillus welchii*, was observed a third pathway for the bioconversion of glycerol. This route consists in the glycerol dehydratation to 3-hydroxypropionealdehyde by means of the enzyme glycerol dehydratase. Subsequently, this intermediary compound is reduced to 1,3-propanediol by 1,3-propanediol oxidoreductase (NADH dependent) [9, 19].

One the glycerol is assimilated into the microbial cell, different compounds can be produced as consequence of its metabolism.

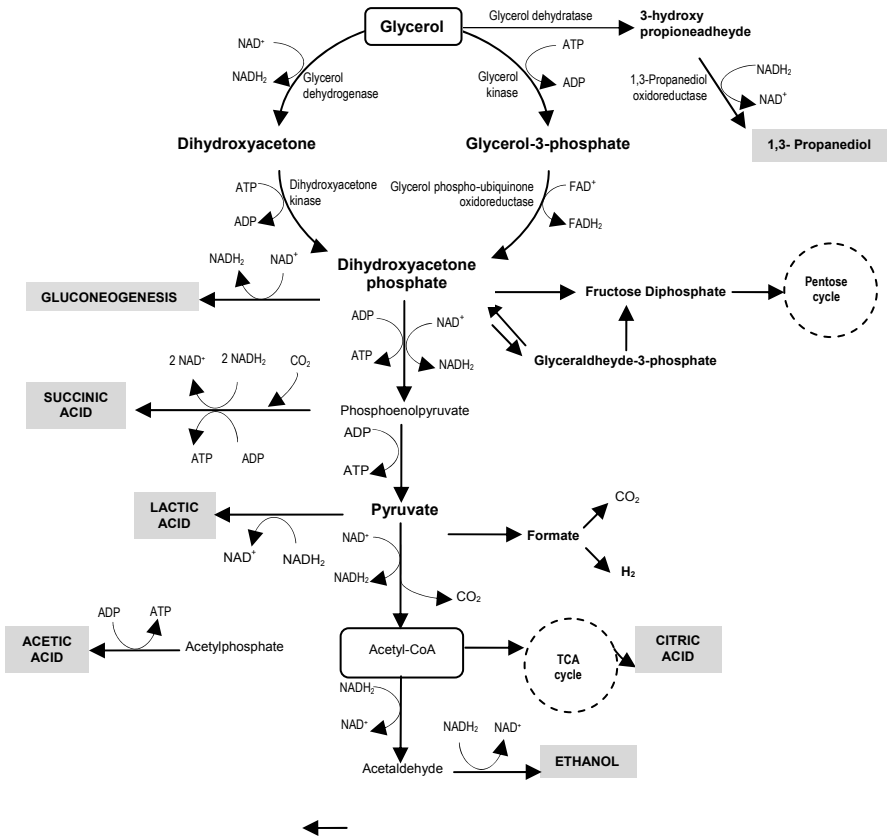

Fig. 1 Pathways of glycerol metabolism under anaerobic and aerobic conditions.

4. Advances on glycerol fermentation in Brazil

Glycerol is considered as a potential renewable low cost substrate to be used in fermentation process for the production of new high-added value bioproducts. Large efforts are being made in order to find new uses for the high volumes of glycerol produced during biodiesel producion. The direct utilization of raw glycerol as principal substrate in fermentation processes can be considered as an important factor in reducing biodiesel production costs and solving part of the pollution problems due to its accumulation. Several bioproducts like 1,3-propanediol, citric acid, succinic acid, ethanol, hydrogen, single cell protein, polyhydroxyalkanoates and biosurfactants, can be produced as strategies for biodiesel valorization.

Recently, it was presented at the XVII Simpósio Nacional de Bioprocessos (National Symposium of Bioprocess, Curitiba, Brazil) the principal researches and new bioprocesses for glycerol utilization. For example, Silva and Contiero (2007) isolated a gram negative bacterial strain GLC29 that can consume 20 g/L of glycerol (as substrate) to produce 11 g/L of 1,3-propanediol, besides, co-products as acetate (0.2 g/L), lactate (0.4 g/L), succinate (0.3 g/L) and ethanol (0.6 g/L). According to previous works carried out in our laboratories, different yeasts are capable of metabolizing glycerol as the only carbon source for biomass production. Yeasts strains like *Kluyveromyces marxianus var. lactis, Candida guillieromondii, Candida tropicalis, Candida batistae, Wickerhamiella cacticola* and *Hansenula anomala* showed an exponential growth on ordinary mineral medium containing 50 g/L of glycerol supplemented with 1.0 g/L yeast extract at 30^{o}C and pH 5.5 [21]. The strains *Kluyveromyces marxianus var. lactis*, *Candida batistae* and *Hansenula anomala* produced microbial biomass with concentration of 6.5, 7.4 and 8.1 g cell/L after 72 h, respectively. *Hansenula anomala* and *Candida tropicalis* were able to produce considerable amount of ethanol with concentrations of 3.6 and 6.6 g/L, respectively. Other biomolecules including citric acid (0.8 g/L- *K. marxianus*), succinic acid (1.4 g/L- *H.anomala*) and acetic acid (1.1g/L- *C.batistae*) were co-produced in low concentrations [21]. Initial experimental results demonstrated the potential application of these yeasts strains in fermentation process for the production of high valued biomolecules for food and pharmaceutical industries.

Several authors are also focusing on the glycerol utilization for biosurfactant production as a feasible alternative for reducing biodiesel production cost [22, 23]. Prieto et al. 2007 obtained “ramnose based lipids” biosurfactant (glicolipid with one or two molecules of ramnose and a chain of fatty acid) by working with

Pseudomonas aeruginosa in a medium containing 20 g/L of glycerol, obtained 0.4 g/L of ramnose with 45 % of emulsification activity on kerosene and reduction on 53 % surface tension of water (STW). Sousa et al. 2007 studied the production of the same biosurfactant by using previously treated glycerol (40 – 80 g/L). It was observed a reduction of 55 % in STW and that the addition of 1,45 g/L $NaNO_3$ had significant effects on the production of this biosurfactant.

Some filamentous fungi use glycerol as the only substrate for producing large variety of metabolites, including different types of pigments. A marked production of red pigments were obtained from *Monascus ruber* in concentration of 5.2 $UDO_{480\ nm}$ (1 UDO_{480nm} = 15 mg/L pigment) using 15 g/L of glycerol [24].

The Lipase enzyme (triacylglycerol ester hydrolase, EC. 3.1.1.3) was produced by a *Bacillus sp* recently isolated from Amazonic environment and identified as BL74. The maximum lipase activity (24.3 IU/mL) was obtained with a medium containing 10 g/L glycerol as sole carbon source [25].

Clavulanic acid, an important compound utilized next with some antibiotics on infections, can be produced from glycerol fermentation by *Streptomyces clavuligerus* [26]. Gutierrez e Costa Araújo (2007) reported a maximum concentration of 60 mg/L from 15 g/L glicerol.

Many of the researches mentioned above are still in their first steps. An important topic that needs to be more investigated is related to the field of biochemical engineering, where different biochemical aspects, such as, aeration, agitation, kinetic study, mass and energy transfer need to be evaluated and studied. A detailed study concerning these aspects also considered as fermentation parameters are essential in the fermentation process, firstly for better understanding of glycerol consumption mechanism by selected microorganisms, secondly for the process optimization always aiming for its future scale-up. As Brazil is projecting an ambitious program for biodiesel production and utilization, consequently, abundant glycerol is accumulating for the next years. The main challenge for the Brazilian government is to give a financial support and incentives for the recent uprising research projects on glycerol utilization, as well as, to provide an easy technology transfer of the new developed processes at laboratory level to an industrial level (biorefinery). Only, the implementation of new strategies, including biotechnology, could turn more feasible the biodiesel production in Brazil.

5. Concluding remarks

This mini-review presents some promising alternatives for utilization of glycerol as carbon source for microbial conversion. Glycerol utilization by microorganisms to produce biomass, protein and organic chemicals can represent a promising alternative to turn sustainable and to add value to biodiesel production. More studies on glycerol fermentation are being conducting in Brazil and other developing countries as an alternative to solve possible pollution problem due to its accumulation in the environment.

Acknowledgements The authors gratefully acknowledge the support of CAPES and CNPq.

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Effect of different physiological stress on flocculation and fermentative capacity of *Saccharomyces cerevisiae* in lager beer

C.S. Leal-Guerra[1], A. Sierra[2], L. C. Damas-Buenrostro[1-2] and B. Pereyra-Alférez[1,*]

[1] Instituto de Biotecnología. Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León. Pedro de Alba y Manuel L. Barragán S/N. Cd. Universitaria. San Nicolás de los Garza, NL. 66450. México
[2] Cervecería Cuauhtémoc-Moctezuma. Ave. Alfonso Reyes 2202 nte. Col. Bella Vista. Monterrey, N L. 64442. México

[*]Corresponding author: bpereyra@fcb.uanl.mx

Flocculation is one of the main determinants in the strain selection for the elaboration of beer and can be altered by numerous factors of stress, since composition of wort until the geometry of fermentation tanks. In this work we evaluated flocculation of two lager yeasts (C820 and C790), modifying the following parameters: profile of sugars, generational age, hydrostatic pressure, starvation and wort gravity. The results are expressed in percentage of variation with respect to the standard condition. Our results indicate that there were no significant differences between both strains, but in the operation variables. When the glucose-maltose proportion was of 10:6 (%), the flocculation decreased 29 and 42 % for C790 and C820, respectively. Another important feature was the ability to metabolize maltose because the strain C790 used only 1.5 %, whereas the C820 metabolized 4.0%. On the other hand, the generational age 9th to 13th generation, in comparison with 1st generation showed an increased flocculation of 23 - 100% for C790 and C820. The variation of the other parameters did not have a significant effect on flocculation. Unlike the flocculation, the fermentation capacity was not affected by evaluated factors, since important parameters such as the alcohol production, cellular viability and vitality, and concentration of diacetyl were similar than standard condition. Our results suggest that flocculation and fermentation is regulated of different way, therefore they offer an opportunity to predict or consider the behavior of the yeast under some stress factors.

1. Introduction

Flocculation is, certainly, one of the most intriguing characteristics and industrially important of brewing yeast. This is defined as asexual aggregation and this happens like a result of an interaction, mediated by calcium, and proteins (floculins) found in the surface of the flocculants cells with carbohydrates (mannan receptors) present in the cell wall, to form clumps and either sediment to the bottom of the tank (in lagers) or by rising to the surface (ale yeast) at the end of beer fermentation. If this process doesn´t works adequately, yeast suspended at the end of the fermentation process may cause problems of filtration, recovery of the yeast to reuse, flavors and odors [14,19]. This process is controlled by four factors: genetic background, mediated by FLO genes, they codify for the adhesion proteins, which they form the family of the floculins [3, 7, 19, 9]; nutritional status, fermentable sugars and other necessary nutrients like a amino acids or free amino nitrogen (FAN), vitamins, minerals, etc. [7]; physiological state: generation number, viability, vitality; and environmental conditions: ethanol, pH, osmotic pressure, etc. [15]. At the moment some conditions of fermentation have increased to rise production of the fermented product, it is the case of the worts with high presence of glucose that has become very important in the last years, due to the high demand of low calories products [5, 7, 14]. On the other hand, osmotic pressure, in the last 20 years the tendency has been "high gravity brewing", that is to say, fermentations using concentrated worts between 12 °P to 20°P [7, 16]. This imposes an additional charge of stress in the yeast that is reflected in slower fermentations, diminished viability and vitality, and occasionally defects in flocculation [6, 10, 13]. Also it has been suggested the starvation per prolonged time affects the capacity to flocculate, and this defect is shown in the subsequent fermentations [17, 13]. It has also been suggested that generational age affects the flocculant capacity. It has been observed that young cells flocculate less than those with greater number of generations [11]. Hydrostatic pressures, brewers today are focusing in increase the volume and height of the fermentation tanks. 20 years ago, the conventional tanks were horizontal cylinders, where height did not exceed the 3 m. At the moment vertical cylindroconical tanks are more than 7,000 hl of capacity and with a height of more than 20 m. This factor affects negatively cellular viability, in addition to alterations in its fermentative behavior [6]. In addition alterations have been observed on the flocculation of some yeast in cylindroconical tanks, whereas others seem not to be affected [12, 8]. In this paper we evaluated the effect of different kinds of stress factors in *Saccharomyces cerevisiae*.

2. Material and methods

2.1 Strains. The *Saccharomyces cerevisiae* lager strains C820, C790 and the nonflocculant strain 7754 (Meyen ex- E.C. Hansen) were obtained from the collection of CCM (Brewery Cuauhtémoc-Moctezuma); the initial concentration of number of cells in suspension was adjusted to 1.2 x 10^6 cells/ml by each degree plate.
2.2 Fermentation. This was made by duplicate for each strain about the four evaluated variables, using 2 extreme conditions (low and high stress).
2.2.2 Wort. It was adjusted the content of Ca^+ (60-80ppm), Zn^+ (up to 0.5ppm for the C790 and up to 0.8ppm for 7754 and C820) and FAN (up to 170ppm in worts with 16°P and up to 150ppm in worts with 12°P). We adjusted first the exact amount of wort, for each repetition by duplicate we used 3,800 ml and was separated in two cylinders of 2 L with the similar amount. The initial concentration of oxygen was adjusted to 8 ppm approximately through to bubbling by 5 minute in a 6 L flask.
2.3 Parameters monitored. Daily for 7 days was reported: the temperature stayed 16°C +/- 1°C, cells in suspension, pH, °P (beer analyzer); in the 7º day of fermentation we monitored the ppm of diacetyl (distillation) and % of alcohol (beer analyzer).
2.3.3 Flocculation test. The yeast was obtained in suspension one day before of the maximum percentage of flocculation in where strain C790 was in 3th day and for strain C820 it was in 5th day. Flocculation tests were carried out essentially as mentioned before [4, 20, 2]. In brief: Cells were washed twice with deflocculation buffer (20 mM citrate [pH 3.0], 5 mM EDTA), and resuspended in the same buffer at a concentration 5.3 x 10^7 cells per ml to give an A_{600} of approximately 1.0. After that, 800µl were transferred to a cuvette (1 cm square) containing 200 µl of 100 mM $CaCl_2$ to a final concentration of 20 mM. The cuvette was inverted several times [20], then was reported the absorbance at 0 and 17 min for the strain C820 and o and 18 min for the strain C790, because at time we obtained similar absorbance difference in two strains. The results were reported as percentage of flocculants cells per time in samples by duplicate.
2.3.3.3 Viability and vitality of the yeast harvested. The % of dead cell was obtained by slide culture according to the method of the ASBC [1]; vitality of yeast was made by the method of acidification power in which the yeast were harvested and centrifuged. Then 9 g washed three times with 50 ml of sterile deionized at 4ºC. Pellet was resuspended in 50 ml of double-distilled water at 25°C. This suspension was diluted with 50 ml water, shaken for 10 min, and we added 5ml of water WAP (water acidification power) and pH was measured 5 times each 2 min. After additional 10 min, we added 5ml of glucose 20.2% (w/v) GAP (glucose acidification power) and each 2 min registry pH values. The two parameters were measured by separated. Mathematical evaluation was done according Van Zandycke et al [18].
2.4 Stress factors. a) Osmotic Stress: the density of extract which part of 16°P, we diluted until reaching a density of 12°P. In order to obtain this, we had to make dilutions with treated water (this water is divided in 50% treated by inverse osmosis and 50% of water filtered by sand filters); **b**) **Profile of glucose: maltose**: wort with a relationship of 1:10 and 10:6 of glucose:maltose, were obtained by use the enzyme amyloglucosidase (Attenuzyme LC™); **c) Hydrostatic pressure**; the yeast was obtained from fermentation tanks with different hydrostatics columns: 1.44 x 10^7 kPa and another one of 1.7 x 10^8 kPa. **d) Starvation**: we were propagated fresh yeast in wort. A part ferment directly without treatment, and the other part treated with starvation in where we harvest by centrifugation and washed three times in sterile water, then it was incubated in sterile water at 25°C on an orbital shaker for 22 h (120 rpm) [12]; **e) Generation number**: was obtained yeast of 1th generation of the strain C790 and C820 and another one of 13th generation for the strain C790 and 9th generation for the strain C820.

3. Results

3.1 Effect of stress factors on flocculation: The effect of different variables was similar within strains, where the higher effect was observed in worts with a profile glucose:maltose de 10:6 than others of 1:10, was obtained a decrement in flocculation about 29 – 42% for C790 and C820, also was obtained a catabolic repression of the sugar maltose in which strain C790 left a remainder of 1,5% whereas C820 was of 4,0% respectively; on the other hand the other variable is the generational age in where high generations show an increase that goes of 23 - 100% with respect to first generations for C790 and C820. The variation within others conditions of stress did not to significant effect (Fig. 1).

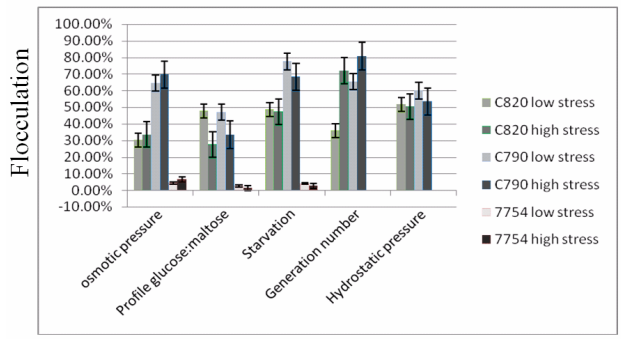

Figure 1: Effect of several stress factors on the flocculation of different brewer's yeast.

3.2 Parameters measured during all fermentations: Cells in suspension reached the maximum growth at day 2 and after it decreased in the two flocculant's strains. The nonflocculant strain reached the maximum growth between days 3 and 4 and main quantity of the cells, were maintained in suspension at the end of the fermentation process (Fig. 2A). Concern to specific gravity (plate degree), the real degree of fermentation, remained around of 60% for all strains (Fig. 2B). Another important feature was those related with the pH, it value decayed since 5.0 to 3.6 at 7th day of the fermentation process in all strains (Fig. 2C).

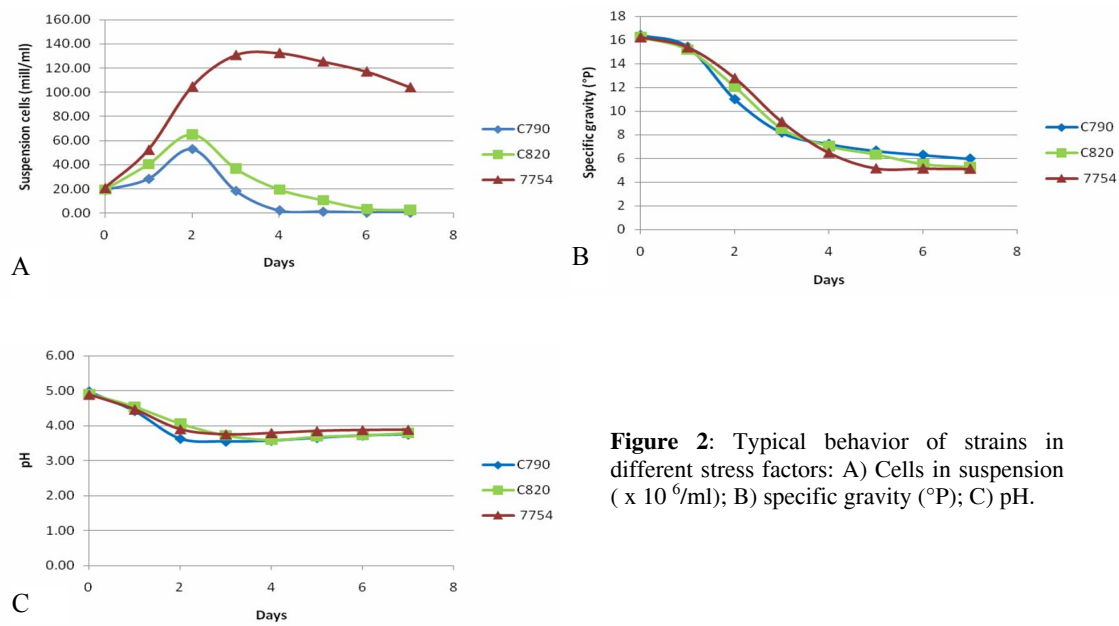

Figure 2: Typical behavior of strains in different stress factors: A) Cells in suspension (x 10 ^{6}/ml); B) specific gravity (°P); C) pH.

On the other hand, the different stress conditions had not significant effect neither on ethanol or diacetyl production, because all strains synthesized standard alcohol concentrations, since 4.7 to 8.1 and 0.1 to 0.6, respectively.

3.3 Viability and vitality of the yeast harvested: The percentage of living cells was more than 94% in all stress conditions for the yeast tested. On the other hand in the acidification power showed a decrement in pH in the WAP test, giving negative values around -0.01 to -0.07; however in the GAP assays the pH was increased, given positive values 0.18 to 0.75 in general.

4. Discussion

It is well known that some stress condition in fermentation process, could to impact negatively on the yeast fitness, such as the flocculation and ethanol production. In this work, we have evaluated the effect of several of them not only the flocculation but also, on ethanol and diacetyl production. The osmotic pressure, starvation and hydrostatic pressure had not negative effect on the flocculation. However, altered glucose:maltose profile of 10:6, instead 1:10, had a negative effect on flocculation phenotype. Early reports suggest that this alteration in flocculation could be due a repressive effect on the FLO11 gene [5]. At the end of fermentation, it was noted that maltose was not totally depleted, it could be due because the glucose cause a repression in MAL genes [6].

On the other hand the stress factor of generation number, shown an increase in the flocculation with old cells (9th to 13th generation) than younger cells (1th generation). Our results are agreeing with Verstrepen *et al* (2003) [19], who suggested that this effect is due because older cells tend to be larger than to younger ones, and to their cells walls are more hydrophobic and wrinkled and they may facilitate the cell to cell adhesion. Another putative reason could be related to the low concentration of flocculins in the cell wall of younger cells. The pH fluctuation and cells in suspension had a normal behavior in all fermentations [7]. On the other hand, real degree of fermentation obtained up to 60%, this indicates that carbohydrates in wort has been successful fermented [6]; Rate of alcohol stayed below 10% (v/v) which to this percentage, the ethanol inhibits brewing yeast growth [7]; in this fermentations diacetyl level stayed in the accepted threshold for lager style; the vitality of strains was measured by two parameters: (GAP) represented the ability of the cells to use extracellular reserves and in this way acidify the medium, in our case, positive values was obtained, and this indicates that the membrane integrity of the three strains was not affected in the high and low parameters of each factor of stress, and on the contrary when the strains were left shaking in water (WAP), exhibited negative values, this shown that our strains had the ability to use their intracellular reserves, like glycogen, to maintain metabolic activity in short-term starvation, and the consumption of this source was minimum [18]. Our results suggest that flocculation capacity and fermentation is regulated by different pathway, therefore they offer an opportunity to predict or consider the behavior of the yeast against some stress factors.

Acknowledgements. This research was support by a grant from Cervecería Cuauhtémoc-Moctezuma. LDB is recipient of a fellow from the CONACYT.

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Evaluation of the β-glucanolytic enzyme complex of *Trichoderma harzianum* Rifai for the production of gluco-oligosaccharide fragments by enzymatic hydrolysis of 1,3;1,6-β-D-glucans

Ellen Cristine Giese[*,1], Alexandre C. Monteiro[1], Aneli de Melo Barbosa[2], Robert F. H. Dekker[3], Osvaldo dos Santos Junior[4], Maria de Lourdes Corradi da Silva[4], Eleni Gomes[1], and Roberto da Silva[1]

[1] Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, São Paulo, Brazil.
[2] Universidade Estadual de Londrina, Departamento de Bioquímica e Biotecnologia, Londrina, Paraná, Brazil.
[3] Universidad de Castilla-La Mancha, Instituto de Regional Investigación Científica Aplicada (IRICA), 13071 Ciudad Real, Spain.
[4] Universidade Estadual Paulista, Faculdade de Ciências e Tecnologia, Presidente Prudente, São Paulo, Brazil.

[*]Corresponding author: e-mail: ellengiese@gmail.com (E.C. Giese)

Botryosphaeran, a new exopolysaccharide from the endophytic fungus *Botryosphaeria rhodina* MAMB-05, and algal laminarin were hydrolyzed by partially-fractionated enzymes of the β-glucanolytic complex from *Trichoderma harzianum* Rifai. β-Glucanase fractions (F-I and F-II) separated by gel permeation chromatography presented different modes of attack on botryosphaeran and laminarin. Botryosphaeran was hydrolyzed to the extent of 66% (F-I) and 98% (F-II) within 30 min, and its main hydrolysis products were gluco-oligosaccharides of DP≥4, with lesser amounts of glucose, di- and tri-saccharides. The action of enzyme fractions I and II on laminarin resulted in 15% conversion to glucose, while the percentage of saccharification was radically different (70% for F-I and 25% for F-II). The different product arrays within the polysaccharide hydrolysates can be explained by the difference in the enzymes' specificities within each enzyme fraction, and the molecular structures of the polysaccharides and their complexity.

Keywords *Trichoderma harzianum* Rifai; non-cellulosic β-glucanases; botryosphaeran; laminarin; gluco-oligosaccharides

1. Introduction

β-1,3-Glucanases are glucan hydrolases of predominantly two types: exo- (EC 3.2.1.58) and endo- (EC 3.2.1.39), and specifically degrade the β-1,3-linked glucosidic linkages of (1→3)-β-glucans and mixed-linked (1→3;1→6)-β-glucans of microbial origin [1]. Fungal β-1,3-glucanases have been used to elucidate the composition and structure of non-cellulosic β-glucans and fungal cell-walls [2], and to produce gluco-oligosaccharides [3,4]. The other hydrolases that complete the key enzymes involved in the degradation of (1→3;1→6)-β-glucans are the β-1,6-glucanases (EC 3.2.1.75) and β-glucosidases (EC 3.2.1.21); each are found in several fungal species [5,6].

Fungal non-cellulosic β-glucans have peculiar biological activities that can include anti-tumor, anti-inflammatory and immune-modulation activities [7]. Increasing the water solubility of these polymers by fragmentation of the polysaccharide chain into shorter chain lengths [8] alleviates the obstacles imposed for their applications. Hydrolysis of β-glucans by microbial β-glucanases presents such an approach, as oligosaccharide fragments of higher degree of polymerization (DP) have been reported to possess preserved biological activity compared with the original parent polysaccharides [9].

The fungus *Trichoderma harzianum* has extensively been used as a bio-control agent as it produces large amounts of enzymes involved in the degradation of the fungal cell wall [10], including β-1,3-glucanases and chitinases. *Botryosphaeria rhodina* MAMB-05, an ascomyceteous endophytic fungus, produces the exopolysaccharide botryosphaeran [11] characterized structurally as a (1→3;1→6)-β-D-glucan [12] (Figure 1), and recently reported as presenting strong antimutagenic activity [13].

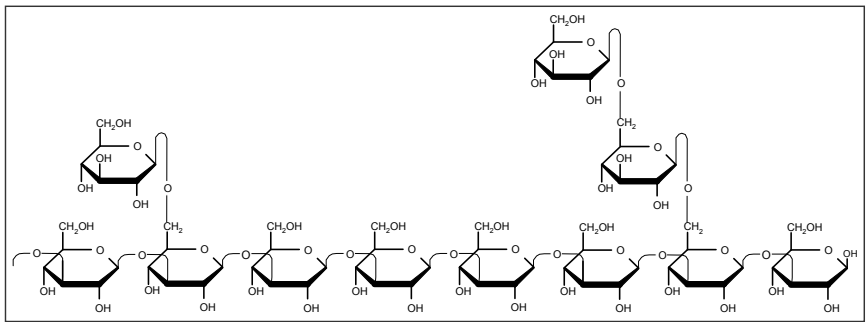

Figure 1. Structure of botryosphaeran, the exopolysaccharide from *Botryosphaeria rhodina* MAMB-05

Considering the commercial importance of obtaining gluco-oligosaccharides of defined chain length through enzymatic hydrolysis of (1→3;1→6)-β-glucans, this work describes the array of hydrolysis products obtained from enzymatic attack on botryosphaeran, and the marine brown algal polysaccharide laminarin, by partially-fractionated enzymes of the β-glucanolytic complex produced by *Trichoderma harzianum* Rifai.

2. Material and Methods

Botryosphaeran was obtained from *Botryosphaeria rhodina* MAMB-05 according to the procedure of Barbosa et al. [12]. Pustulan, isolated from the lichen *Actinogyra muehlenbergii* [14], was kindly donated by P.A.J. Gorin, Universidade Federal de Paraná (Deptº de Bioquímica), Brazil. Laminarin (*Laminaria digitata*), laminaribiose (**G_{2L}**), laminaritriose (**G_{3L}**), gentiobiose (**G_{2G}**) and *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) were purchased from Sigma-Aldrich. For enzyme production, conidia were harvested from cultures of *T. harzianum* Rifai grown on xylose-agar-Vogel minimal salts medium (VMSM [15]) plates over 7 days and used to inoculate (1 x 10^8 spores/flask) liquid medium comprising VMSM and botryosphaeran (1.5 g/l) as sole carbon source. Cultures were incubated at 28 °C on a rotary shaker at 180 rpm for 5 days. Extracellular fluid (ECF) was recovered following centrifugation (7000 x *g*/10 min) at 4 °C, exhaustively dialyzed against de-ionised water, and used as the source of enzyme.

The ECF was pooled, lyophilized and applied to a column of Sephadex G-100 (1.5 x 90 cm) and eluted with 20 mM sodium acetate buffer (pH 4.5) at a flow rate of 15 ml/h. Fractions of 2.5 ml were collected and analyzed for β-1,3-glucanase, β-1,6-glucanase and β-glucosidase activities. β-Glucanase activity was determined against the polysaccharides laminarin (4 g/l; a β-1,3-glucan) and pustulan (2 g/l; a β-1,6-glucan) in a final volume of 0.5 ml that comprised enzyme (ECF) and 25 mM sodium acetate buffer (pH 4.5). The hydrolysis products released were measured as reducing sugars by the cuproarsenate method of Nelson [16] and Somogyi [17]. β-1,3-Glucanase activity was measured at 50 ºC for 10 min, and β-1,6-glucanase activity at 50 °C for 20 min. The unit of each β-glucanase activity was defined as the number of μmol reducing sugars produced/min/ml. β-Glucosidase activity was measured with *p*-NPG (4 g/l) as substrate, and was incubated at 55 °C for 20 min [18]. The unit of β-glucosidase activity was defined as the number of μmol *p*-nitrophenol liberated/min/ml.

Laminarin and botryosphaeran (1 g/l) hydrolysis was conducted in 20 ml solution (pH 5.0) using 1.0 unit of each enzyme fraction (activity measured against laminarin) at 40 ºC. The hydrolysis products were measured by the reducing sugar method, total sugars by the phenol-sulfuric acid method [19], and analysed by High Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC/PAD). Glucose was measured by the glucose oxidase method using a Glicose Enz Color reagent kit (Bio Diagnóstica, Curitiba, Brazil).

3. Results and Discussion

3.1 Enzymatic hydrolysis of botryosphaeran

β-1,3-Glucanases have been used to depolymerise (1→3;1→6)-β-glucans to obtain degraded fragments possessing biological activities such as prebiotic, pharmaceutical and functional compounds [9,20,21]. In this work, we examined the enzymatic hydrolysis of botryosphaeran and laminarin to understand the nature of the reactions in hydrolysing (1→3;1→6)-β-glucans, and the products released.

The hydrolysis of botryosphaeran by partially-fractionated enzymes of the β-glucanolytic complex (F-I) from *T. harzianum* Rifai grown on botryosphaeran as sole carbon source, resulted in 66 % saccharification in 30 min that corresponded to 68 % of gluco-oligosaccharides with DP ≥4 as products by HPAEC/PAD analysis. By contrast, the F-II enzymatically-degraded botryosphaeran was less extensively degraded over the same hydrolysis period, with a degree of hydrolysis of ~10 % at 30 min. By 30 min, hydrolysis by F-II leveled-off and hydrolysates showed minor quantities of glucose, gentiobiose, laminaribiose and laminaritriose. A time course profile for the hydrolysis of botryosphaeran by the two enzyme fractions (F-I and F-II) under the chosen conditions revealed that hydrolysis was linear over the 30 min.

Crude enzyme preparations from *T. harzianum* have produced a broad range of products from botryosphaeran in short incubation times with only tetrasaccharides as the highest DP [3]. In the present work, gentiobiose and laminaribiose appeared in approximate proportions (Figure 2), and laminaritriose was present in the hydrolysates in very small amounts. F-I revealed 1,3-β-glucanase, 1,6-β-glucanase and β-glucosidase activities, while F-II only demonstrated action on (1→3;1→6)-β-glucans.

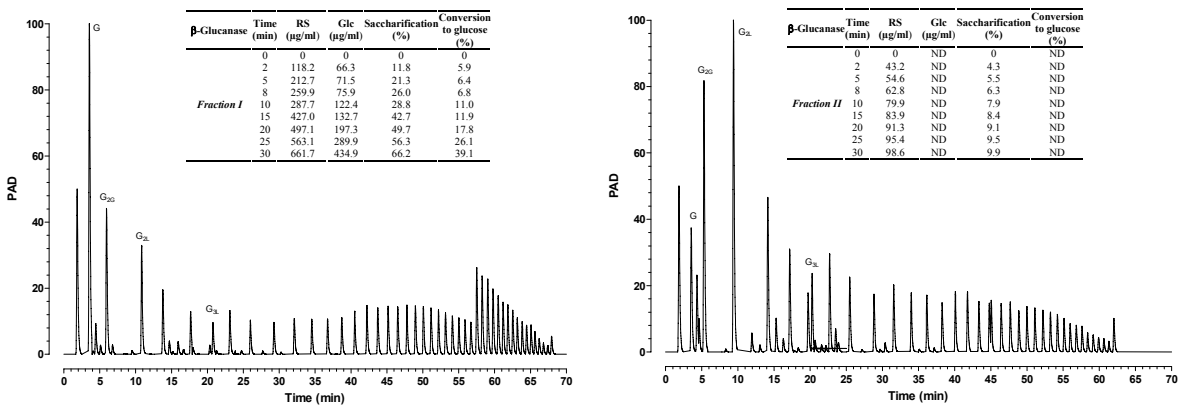

Figure 2. HPAEC/PAD analysis of hydrolysis products arising from botryosphaeran through the action of partially-fractionated enzymes of the β-glucanolytic complex from *Trichoderma harzianum* Rifai. Hydrolysis conditions: β-1,3-glucanase 1.0 U; 40 °C, 30 min. RS, reducing sugars; Glc, glucose; ND, not detected.

By comparison, the product array from the enzymatic hydrolysis of botryosphaeran by F-II was very different. Approximately only 10 % of botryosphaeran was hydrolyzed within 30 min. A low amount of glucose was detected during the stages of hydrolysis, as well as gentiobiose, and only small amounts of laminaribiose and laminaritriose were detectable by HPAEC/PAD analysis. Major oligosaccharides were detected, however, to a lesser degree than those detected through the action of F-I.

3.2 Enzymatic hydrolyzes of laminarin

At the stage of 30 min hydrolysis, ~70 % of laminarin had been hydrolysed through the action of F-I, which represented a conversion into glucose of 15 %. The high degree of hydrolysis was representative of oligosaccharides presenting DP ≥4. HPAEC/PAD analysis revealed a difference in the product array from the hydrolysis of laminarin for each enzyme fraction (Figure 3). The trend in hydrolysis of laminarin resulted in a lower degree of conversion into glucose during 30 min. Gluco-oligosaccharides with DP ≥4 were the major hydrolysis products, followed next by gentiobiose and minor amounts of oligosaccharides of DP ranging from 2 to 4. In separate experiments, some enzymatic hydrolysates were spiked with the authentic sugar standards to identify that the peaks with similar retention times were in fact those products identified by HPAEC-PAD. No anomalies were observed in the spiked samples, and glucose, gentiobiose, laminaribiose and laminaritriose were positively identified in the hydrolysates by this technique as was also earlier observed by Giese et al. [3]. The product array in hydrolysates of laminarin by F-II was similar to that obtained by F-I, but the amounts of gluco-oligosaccharides of DP ≥4 were vastly greater. Some tetrasaccharides and possibly pentasaccharides also appeared but were not identified.

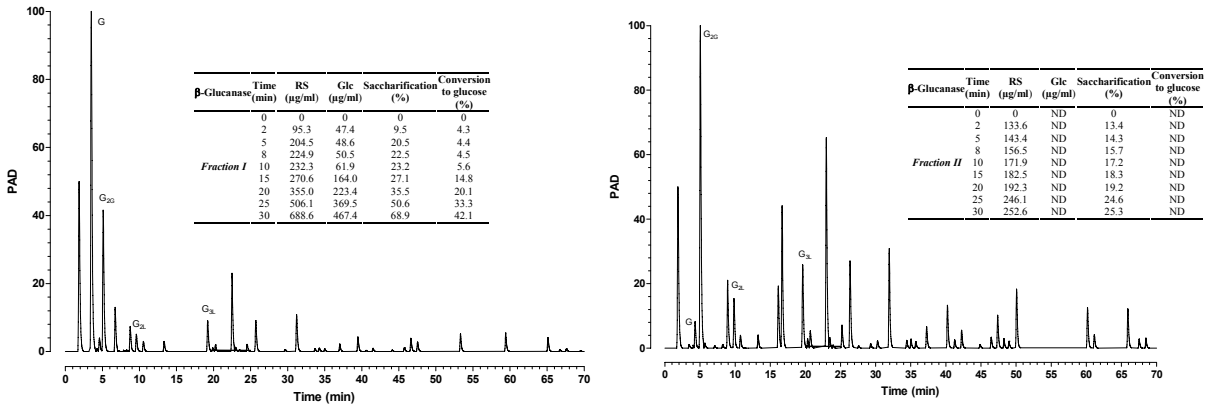

Figure 3. HPAEC/PAD analysis of hydrolysis products arising from laminarin through the action of partially-fractionated enzymes of the β-glucanolytic complex from *Trichoderma harzianum* Rifai. Hydrolysis conditions: β-1,3-glucanase 1.0 U; 40 °C, 30 min.

The action of partially-fractionated enzymes of the β-glucanolytic complex from *T. harzianum* Rifai on laminarin was somewhat different than towards botryosphaeran, and reflects a difference in the molecular structure between the two polysaccharide types, and the complexity of botryosphaeran due to ramifications at C-6 along the β-1,3-glucan chain. Botryosphaeran is more highly branched (22 %) with glucose and gentiobiose units [22], while laminarin is ramified to the extent of 5-10 % with single glucose units [23]. The specificity of the different β-glucanolytic enzyme fractions too will have an effect on the product array in hydrolysis of botryosphaeran and laminarin.

Acknowledgements Support from CAPES, FAPESP and Fundação Araucária do Paraná-Brasil is gratefully acknowledged.

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Growth of *Kluyveromyces marxianus* yeasts strains in deproteined whey obtained from dairy industry

Natalia Oliveira Calil[1], Humberto Moreira Húngaro[1], Flavio Oliveira Ferraz[1], Aline Siqueira Ferreira[1], Silvio Silvério da Silva[1,2,3]

[1] Department of Food and Toxicology, Faculty of Pharmacy, Federal University of Juiz de Fora - Juiz de Fora, MG – Brazil
[2] Departament of Biotechnology (DEBIQ), University of São Paulo – EEL-USP - Lorena-SP-
[3] Brazil Faculdades Integradas Teresa D'Ávila – FATEA – Lorena – SP – Brazil

Introduction

Biotechnology has been contributing significantly to the generation of added value products and processes with recognized viability, showing high application, mainly in food and pharmaceutical industries [4].
Moreover, it has an important role in reduction of environmental impact caused by some industrial pollutants, as sugar cane bagasse, rice and oats straw, coffee hulls, hulls and seeds of fruits, cheese whey, lipidic waste of edible oils, and other. All of this due to its use in fermentation processes to obtain products of commercial interest, as sugars, ethanol, dyes, essential oils, bioactive compounds, proteins, enzymes, bioinseticides, biopolymers and biodiesel production [9;14].

Among these by-products, highlights to cheese whey due to its high carbohydrate rate, composed mainly by lactose, that can be used as energy and carbon source by microorganisms that are able to assimilate this sugar.
Many microorganisms are able to produce β-galactosidase, responsible for lactose hydrolysis, and assimilation of this carbohydrate as the main source of carbon in fermentative processes [13].

Kluyveromyces marxianus is a unicellular microorganism, from ascomicetos class, that presents easy assimilation of lactose, being widely used in preparations of dairy products, mainly fermented beverages based on cheese whey and milk [2; 11]. This yeast strain is responsible for many products made from cheese whey, with the large scale production of single-cell protein [5], aromatic compounds, biologically active secondary metabolites, enzymes, organic acids, amino acids [7] and ethanol [6].

Many efforts have been done to use cheese whey as growth medium to the production of biomolecules with high added value. However in the most of cases occurs the supplementation of the media with elements and yeast extract, in order to provide the nutritional requirements of the employed microorganism [1; 12; 8].

The main aspect of this investigation was to evaluate the growth of two strains of *Kluyveromyces marxianus* in deproteined and sterilized cheese whey, and assess the need of supplementation of this medium for high biomass production.

Material and methods

Experiences were done using cheese whey from enzymatic coagulation of *minas frescal* (a typical Brazilian cheese) manufacture, previously deproteinized by acidification and warming, followed by cooling and filtering of the protein, and then sterilized at 121ºC for 15 minutes.

It was evaluated growth of two strains of yeast *Kluyveromyces marxianus* (ATCC 8554 e CCT 4294) in deproteined and thermally treated whey, supplemented and not supplemented to verify the need of addition of nutrients to the culture medium.

The inoculum was prepared using a suspension of each yeast Kluyveromyces marxianus ATCC 8554 or CCT 4294 in physiological solution with 1.6% of transmittance read in spectrophotometer at λ = 600 nm, and added 0.1 mL of these suspensions as inoculum.

Cultivation of each yeast cell were done in 250 mL erlenmeyer flasks containing 100 mL of whey, supplemented with 0.1 g / L yeast extract, 1.0 g / L ammonium sulfate and 1.0 g / L potassium phosphate monobasic, by beforehand prepared solutions. Control experiences with not supplemented whey were done at the same experimental conditions. All the experiments were carried out in triplicate.

Samples were incubated at 30 ° C over a period of 48h. After this period, the biomass production were evaluated through the drying of an aliquot of 10 mL of samples at 105 °C and the dry weight was determinated.

Results and discussion

Table 1 and 2 show the biomass formation in deproteined whey supplemented and not supplemented for the two yeast strains studied.

Table-1. Biomass obtained through fermentation of deproteined whey with *Kluyveromyces marxianus* ATCC 8554.

Medium culture	Sample	Biomass (g/L)
Suplemented	A1	31,58
	A2	30,42
	A3	27,26
Not suplemented	A4	33,08
	A5	28,54
	A6	28,77

Table-2. Biomass obtained through fermentation of deproteined whey with *Kluyveromyces marxianus* CCT 4294.

Medium culture	Sample	Biomass (g/L)
Suplemented	B1	36,38
	B2	37,10
	B3	33,50
Not suplemented	B4	32,07
	B5	34,11
	B6	30,17

After 48h of incubation time there was considerable production of biomass of both yeasts used, even in supplemented or not supplemented cheese whey.

Although the yeast *Kluyveromyces marxianus* CCT 4294 present values of biomass slightly higher than the other strain, after processing the data, there were found out that there was no significant statistical difference (95% confidence) between the results.

Comparing statistically (parametric test, BONFERRONI) the results of biomass obtained, between the whey supplemented and non-supplemented, there was not statistically significant difference ($p > 0.05$) between the samples and it is considered a level of significance of 5% .

In most studies [6; 8; 12; 10; 1; 3] using cheese whey as growth medium for yeast *Kluyveromyces marxianus*, the authors added solutions of salts in the culture medium as a source of sulfate, nitrogen, phosphate, magnesium and potassium, as well yeast extract and meat peptone in order to enrich the culture medium and improve the cell growth.

This study demonstrated that there is no need of cheese whey supplementation with addition of solutions of ammonium sulfate, potassium phosphate and yeast extract at the concentrations used for the growth of the yeasts in study.

Compared with the work of the other cited authors, not supplementation of the cheese whey makes simpler the fermentative process and makes it feasible for optimization and production of biomass on a large scale.

Conclusion

From the results obtained, it can be concluded that both strains of yeasts used grown in supplemented or not supplemented cheese whey, showing no statistically significant differences on the need for supplementation.

Thus, the cheese whey presented applicability as a culture medium in fermentative processes using the yeast *Kluyveromyces marxianus* for production of biomass and biomolecules of commercial interest.

Acknowledgments: FAPEMIG, CnPQ.

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Isolation and characterization of α-glucosidase — a thermostable intracellular enzyme from *Aspergillus carbonarius* var (bainer) Thom IMI 366159

C.O. Onwosi[1,*]

[1] Biotechnology Research Centre, Nnamdi Azikiwe University, P. M. B .5025, Awka, Anambra State, Nigeria.

[*]Corresponding author: onwosicoach@yahoo.com , +2348034920577

Aspergillus carbonarius var (bainer) Thom IMI 366159 produced a cell bound α -glucosidase when grown on PDA. Time course study was carried out to determine the maximum activity of the enzyme which occurred after 72h (3.29U/ml) after shaking for 144h in a rotary shaker. The enzyme assay revealed that the optimum temperature for the activity and stability were 60^{o}C and 50^{o}C respectively. The enzyme was stable at pH 4.5-5.0 and had optimum activity of 4.2U/ml at pH 3.5. The crude enzyme preparation also hydrolysed p-nitrophenyl α-D- glucopyranoside (PNPG) to produce a yellowish p-nitrophenol. This marked its substrate specificity for α -1,6-1inkages. It is also suggested that the enzyme was produced intracellularly as it has low activity in cell free supernatant.

1. Introduction

A number of enzymes are associated with degradation of starch and/or glycogen. Starch or its hydrolysate is an excellent and widely used raw material for many fermentation processes. The polysaccharide is used directly by many microorganisms, by virtue of their ability to produce starch hydrolyzing enzymes [1].

In other instances, hydrolysates in form of glucose and/or dextrin-which are prepared from starch by isolated enzymes-are used as fermentation substrates. Microbial enzymes of commercial significance in relation to starch technology include: amylase, an extracellular enzyme of specific for α -1, 4, linkages and capable of by passing α -1, 6-linkages; β amylase, an extracellular or intercellular enzyme that appears to hydrolyse α- 1, 4 and/or α -1**,** 6 linkages in short chain saccharides arising from the action of other enzymes of starch. Microbial α -glucosidases (α - D-glucosideglucohydrolase) hydrolyse terminal, non reducing α -1, 4 linked glucose residues in different substrates, releasing α -D-glucose. Most of the microbial and α amylases belong to the family 13 glycosyl hydrolases, and they share several properties [3]. α -glucosidase [2] is one of the most important members and amylase family α - glucosidases in nature and it is orderly distributed in microorganisms, animal and plant kingdom[3,4]. It is generally found in association with amylases. The enzyme also occurs in considerable number of microorganisms-bacteria, mould, yeast and fungi.

2. Materials and method

2.1 Organism and growth condition

Aspergillus carbonarius var (bainer) Thom IMI 366159 was used. It was grown on enriched medium with the following composition: soluble starch, 16g; glucose, 0.49g; KH_2PO_6, 1.29g; $MgSO_4$. $7H_2O$,0.49g; NaCl, 0.049g, $FeSO_4.7H_2O$,0.04g; $ZnCl_2$, 4µg; $MnSO_4.7H_2O$,4µg; $CuSO_4.7H_2O$, 4µg; yeast extract, 2g; casamino acid ,2g and deionized water 400ml [5] The medium was autoclaved at 121^{o}C, 20minutes 15lbs. The inoculum was prepared by growing cell on potato dextrose agar (PDA) for three days. The spores of the inoculum were their suspended in 10ml of deionised water containing 1 drop of surfactant (tween 80). The 1ml of the suspension is inoculated into each of the eight (200ml) Erlenmeyer flask containing 80ml of the medium. The flasks were incubated in an orbital shaker al 200rpm at 37^{o}C for six days. After each day, one flask is removed for assay for enzyme activity.

2.2 Measurement of cell dry weight

Cells isolated from 0.4l of culture by centrifugation (10000gxg for 15 mins) were washed twice in 0.1m potassium phosphate buffer (pH6.0). The dry weight of the cells was determined by drying standard aliquots taken at various time intervals in an oven at 90^{o}C and subsequently weighed in balance after drying.

2.3 Enzyme assay

α-glucosidase activity was assayed according to the method of Plant *et al.* [6] by measuring the release of p-nitrophenol from P-nitrophenyl α -D glucopyranoside (PNPG). The assay mixture, consisting of 200ml PNPG of cell free supernants and 2ml of 2mM PNPG in 20mM potassium phosphate buffer pH 6.0, was incubated at 37^{o}C for 30 minutes. Aliquots of 500ml were taken at the start, and at end of incubation were added to 500µl of 1M Na_2CO_3 solution, and at the absorbance were measured at 420nm.
One unit (U) of α-glucosidase activity is defined as amount of enzyme causing an increase in absorbance of 420nm of 0.1 in 1hour under reaction condition.

2.4 Biophysical characterisation

2.4.1 Effect of temperature activity and stability of α -glucosidase

The effect of temperature on enzyme activity was determine by carrying out the standard enzyme assay for α -glucosidase at a range of temperature between 30-90^{o}C with 10^{o}C increments using 0.2ml of the enzyme. On the effect of temperature on stability, 200ml of the enzymes were exposed to various temperatures as described above for 30minutes. After which the enzyme was chilled and normal enzyme assay was carried out at 37^{o}C (usual assay temperature).

2.4.2 Effect of pH on α -glucosidase activity and stability

100mM of potassium phosphate buffer solution of different pH values (3.5-7.5) with 0.5 increments were prepared. For pH activity, equal volume of buffer was added to equal volume of enzyme solution and enzyme activity determined at assay temperature. On the effect of pH on α -glucosidase stability equal volumes of enzyme and buffer were mixed as described above. Incubation was done at optimum temperature for about 2hours. After which, the normal assay for enzyme activity was done at normal assay temperature.

3. Results and discussion

α - glucosidase was produced by *Aspergillus carbonarius* var (bainer) Thom IMI 366159. The time course study revealed that after 144h shaking, the best level of α -glucosidase production occurred after 72h (table 1). The highest enzyme activity (3.29U/ml) correlated with cell dry weight (1.039g) and this occurred in the third day (72h) during the time course study on the enzyme production. Schiraldi *et al*. [7] reported the effective production of a thermostable α-glucosidase from *Solfolobus solfataricus* in *Escherichia coli* exploiting a microfilteration bioreactor. Castro *et al*. [8] used *Bacillus* sp strains in production of α-glucosidase. Sato *et al.* [4] produced glucoamylase a typical α-glucosidase-from *Schwanniomyces occidentalis.*

Aspergillus carbonarius at different pH shows an optimum activity at pH 3.5. The stability of the enzyme is also maximum at pH range of 4.5 -5.0 (table 2). This was within the pH range of 4 -11 reported earlier [15, 9, 10, 11, 12]. However; α amylases with stability in a narrow range have also been reported. [13]. Gomes *et al*. [14] reported 100% stability at pH range of 5-9 for *Aspergillus flavus*.

The optimum temperature for activity of the enzyme occurred at 60^{o}C while the stability of α- glucosidase was at temperature of 50^{o}C (table 3). Vihinien *et al.* [9] have reported that the temperature optimum for the activity of α amylase is related to the growth of microorganism involved. Gomes et *al.* [14] equally reported a themostability temperature of 60^{o}C for *Aspergillus flavus*. Thermostabilities are affected by many factors like presence of calcium, substrate and other stabilizers [9].

Table 1 A time course study for the production of α -glucosidase

Time (h)	Activity (U/ml)	Cell dry mass (g)
24	0.19	0.016
48	0.25	0.3
72	3.29	1.03
96	2	0.65
120	1.5	0.49
144	0.6	370

Table 2 Effect of pH on the relative activity and stability of α- glucosidase

pH	Relative activity (%)	Relative stability (%)
3	95	20
3.5	100	50
4	81	60
4.5	62	100
5	48	100
5.5	43	80
6	33	70
6.5	24	40
7	19	30

Table 3 Effect of temperature on relative activity and stability of α- glucosidase

Temperature	Relative activity (%)	Relative stability (%)
30	2	20
40	17	20
50	42	100
60	100	38
70	52	20
80	33	2
90	8	0

4. Conclusion

α- glucosidase was reported specific for α 1, 6- linked sugar since it attacked PNPG. The enzyme is produced intracellularly because it has low activity in cell free supernatant and thus can be involved in the endogenous glucose metabolism as the final enzymes required to hydrolyze low molecular weight saccharides transported into the cell via permeases [15]. The enzyme is stable under high temperature and acid pH range is required for the enzyme activity and stability. This suggests that the enzyme can be applied in many industrial fermentation requiring high temperature and acidic pH.

Acknowledgements The support by A.I Ikeyina and Sons, ltd. is gratefully acknowledged.

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Purification and biochemical characterization of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb. :Fr.) Rehm

A. Karnchanatat[*],[1] A. Petsom[2], P. Sangvanich[2], J. Piaphukiew[3], A. J. S. Whalley[4], C. D. Reynolds[4], P. Sihanonth[5]

[1] Biotechnology Programme, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[2] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[3] Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[4] School of Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, U.K.
[5] Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand

[*]Corresponding author: email: i_am_top@hotmail.com, Phone: +6681-567-5568

An extracellular β-glucosidase was purified from culture filtrates of the wood decaying fungus Daldinia eschscholzii (Ehrenb.:Fr.) Rehm grown on 1.0% (wt/vol) carboxymethyl-cellulose using ammonium sulfate precipitation, ion-exchange, hydrophobic interaction, and gel filtration chromatography. The enzyme is monomeric with a molecular weight of 64.2 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and has a pI of 8.55. The enzyme catalyzes the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate, with a Km of 1.52 mM, and Vmax, of 3.21 U/min/mg protein. Glucose competitively inhibited β-glucosidase with a Ki value of 0.79 mM. Optimal activity with PNPG as the substrate was at pH 5.0 and 50 °C. The enzyme was stable at pH 5.0 at temperatures up to 50 °C. The purified β-glucosidase was active against PNPG, cellobiose, sophorose, laminaribiose, and gentiobiose, but did not hydrolyze lactose, sucrose, Avicel or o-nitrophenyl-β-D-galactopyranoside. The activity of β-glucosidase was stimulated by Ca2+, Co2+, Mg2+, Mn2+, glycerol, DMSO, DTT and EDTA, and strongly inhibited by Hg2+. The internal amino acid sequences of D. eschscholzii β-glucosidase have similarity to the sequences of the family 3 β-glucosyl hydrolase.

Keywords β-glucosidase, wood-decaying fungus, *Daldinia eschscholzii*

1. Introduction

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of β-glucosidic linkages, such as those in alkyl- or aryl-β-glucosides as well as diglucosides and oligosaccharides. They represent an important group of enzymes because of their potential use in various biotechnological processes, including biomass degradation [1], production of fuel ethanol from cellulosic agricultural residues [2], release of aromatic compounds in the flavour industry [3], and synthesis of useful β-glucosides [4]. A complete set of cellulolytic enzymes is minimally composed of the following components: endoglucanases (endo-β-1,4-glucanase, EC 3.2.1.4), exoglucanases (exo-β-1,4-glucanase, EC 3.2.1.91), and β-glucosidases [5]. The role of the β-glucosidase in the saccharification of cellulose is to degrade cellobiose, an inhibitor of the depolymerizing enzyme, and cellulooligosaccharides to glucose. However, β-glucosidase is frequently a rate-limiting factor during enzymatic hydrolysis of cellulose and is very sensitive to glucose inhibition which limits its activity [6].

The *Xylariaceae* are wood-decaying fungi found in terrestrial habitats, are particularly diverse in tropical regions, and are able to degrade lignocellulose. Many of the *Xylariaceae* are saprotrophs on decaying plant material such as logs and stumps, dead twigs and branches of trees, dead leaves and stems of herbaceous plants [7]. It is also clear that many species can degrade lignin [8] and that others exhibit impressive production of cellulolytic enzymes [9]. *Daldinia* (*Xylariaceae*) is a genus of wood-inhabiting pyrenomycetes with perithecia embedded in large stromata that are internally concentrically zoned and produce enzymes that digest the cell wall components for nutrition and energy [10]. In this paper, we report on the purification and detailed biochemical characteristics of an extracellular β-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. It is noted that the activities of *D. eschscholzii* have not as yet been reported.

2. Materials and method

2.1 Organism and culture conditions

The specimen was collected from a dead mango tree in the Royal Forest Department park, Bangkok during June 2003. The fruiting structure was identified as *Daldinia eschscholzii* based on morphological characteristics and then confirmed using molecular techniques. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB284189.The stock culture of *D. eschscholzii* was maintained on potato dextrose agar medium. Unless otherwise stated, actively growing fungal mycelium from a 7 day old culture was transferred to a 250-ml Erlenmeyer flask containing 100 ml of Mandels medium [11] composed of; urea 0.3 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2.0 g/L, $CaCl_2{\cdot}2H_2O$ 0.4 g/L, $MgSO_4{\cdot}4H_2O$ 0.3 g/L, peptone 1.0 g/L, $FeSO_4{\cdot}7H_2O$ 5.0 mg/L, $MnSO_4{\cdot}4H_2O$ 1.6 mg/L, $ZnSO_4{\cdot}7H_2O$ 1.4 mg/L, $CoCl_2{\cdot}6H_2O$ 2.0 mg/L, and Tween 80 2.0 ml/L, pH 5.5 to which 1% (wt/vol) of various carbon sources such as Avicel® PH-101 (Fluka), Carboxymethyl-cellulose (Fluka), Filter paper (Whatman No.1), and glucose (Fluka) were added. The medium was sterilized by autoclaving at 121 °C for 20 min. Inoculated flasks were incubated on a rotary shaker (New Brunswick Scientific) at 150 rpm at 25 °C for 14 days under natural light conditions. The culture fluid was filtered through filter paper (Whatman No.1) and the supernatant fluid was used as the crude enzyme preparation.

2.2 Assay of β-glucosidase

β-Glucosidase activity was determined by measuring the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside (PNPG) as described previously [12]. The reaction mixture (1 ml) contained 5 mM PNPG (Sigma) in 0.1 M sodium acetate buffer (pH 5.0) and an appropriately diluted enzyme solution. After incubation at 50 °C for 30 min the reaction was stopped by adding 1.0 ml ice-cold 0.25 M Na_2CO_3 and the colour formed was measured at 410 nm (Tecan Sunrise). One unit of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmole of *p*-nitrophenol per min under the assay conditions. Specific activity is defined as the number of units per milligram of protein.

2.3 Purification of β-glucosidase

2.3.1 $(NH_4)_2SO_4$ Precipitation

To 5 liters of culture supernatant $(NH_4)_2SO_4$ was added to give 80% saturation. After standing overnight the precipitate formed was collected by centrifugation at 10,000 g for 20 min (Beckman Coulter), dissolved in 20 mM sodium acetate buffer, pH 5.0. The dissolved sample was dialyzed against the same buffer and concentrated by lyophilization (Labconco).

2.3.2 Cation exchange chromatography

The sample solution was applied on a column (1.6 × 10 cm) of a SP Sepharose Fast Flow (Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0. Elution was undertaken with the same buffer at a flow rate of 1.0 ml/min. A linear gradient of 0-1.0 M NaCl in the same buffer was then applied. Fractions of 10.0 ml each were collected and assayed for β-glucosidase activity. The fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

2.3.3 Hydrophobic interaction chromatography

To the active fraction from the SP Sepharose Fast Flow, $(NH_4)_2SO_4$ was added to a concentration of 30%. The mixture was applied to a column (1.6 × 10 cm) of Phenyl Sepharose Fast Flow (Amersham Biosciences) equilibrated with 30% $(NH_4)_2SO_4$ in 20 mM sodium acetate buffer, pH 5.0. The column was then eluted with a gradient of 30-0% $(NH_4)_2SO_4$ in 20 mM sodium acetate buffer, pH 5.0 at a flow rate 1.0 ml/min. Fractions of 5.0 ml were collected and assayed for β-glucosidase activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further purification.

2.3.4 Gel Filtration Chromatography

The active fraction from Phenyl Sepharose Fast Flow was applied to a column (1.6 × 60 cm) of Superdex 200 HR (Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer, pH 5.0 containing 100 mM NaCl at a flow rate of 1.0 ml/min. Fractions of 5.0 ml were collected and assayed for β-glucosidase activity. The active fractions containing β-glucosidase activities from the column were pooled and dialyzed against the same buffer for further analysis.

2.4 Protein determination

Protein concentrations in the enzyme preparations were determined by the method of Bradford [13] with reference to a standard calibration curve for bovine serum albumin (BSA). During the column chromatographic separations the elution profiles of proteins were determined by measuring absorbance at 280 nm.

2.5 Molecular weight and isoelectric point determination

2.5.1 SDS polyacrylamide gel electrophoresis

The gel was prepared with 0.1% SDS in 12.5% separating gels and 5.0% stacking gels. Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli [14]. Samples to be analyzed were treated with sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run from the cathode to anode at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Amersham Biosciences). High and low molecular weight standards (Sigma) were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie Blue R-250 [14].

2.5.2 Isoelectric focusing polyacrylamide gel electrophoresis

IEF-PAGE was performed on the Phast-System of Pharmacia LKB using a precast gel, PhastGel IEF 3-9. Isoelectric points were determined using standard pI markers (BioRad).

2.6 Effect of temperature on β-glucosidase activity and stability

The optimum temperature for enzyme activity was determined by monitoring each activity at pH 5.0 at various temperatures from 30 °C to 80 °C. Stability was measured by incubating the enzyme in 0.1 M acetate buffer pH 5.0 for 30 min at temperatures from 30 °C to 80 °C. Following incubation, the enzyme solution was cooled, and the remaining activity was determined under standard enzyme assay conditions [12].

2.7 Effect of pH on β-glucosidase activity and stability

The optimum pH of activity was determined by monitoring each activity at 50 °C at various pH values ranging between 3.0 to 9.0. The following buffers were used: 0.1 M sodium acetate buffer (pH 3.0-6.0); 0.1 M phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.0-9.0). The β-glucosidase stability was examined at the pH values 3.0-9.0. Enzyme samples were pre-incubated in the above-cited buffers at 30 °C for 1 h before adding the substrate. After adjustment of the pH the residual activity was determined under standard enzyme assay conditions (12).

2.8 Effect of metals and reagents

The effects of various metal ions and reagents at 1 mM on β-glucosidase activity were determined by preincubating the enzyme with the individual reagents in 0.1 M sodium acetate buffer pH 5.0 at 30 °C for 30 min. Activities were then measured at 50 °C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

2.9 Substrate specificity

Several α- and β-glucosides (20 mM), saccharides (1%, wt/vol), and arylglycosides (5 mM) were tested as substrates for the purified enzyme. The p-nitrophenol released was determined under standard enzyme assay

conditions [12]. The total amount of reducing sugars (expressed as equivalent glucose) in 1.0 ml supernatant was determined by the modified dinitrosalicyclic acid (DNS) method [15].

2.10 Determination of kinetic parameters

The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for β-glucosidase by incubating in 0.1 M sodium acetate buffer pH 5.0 at 50 °C with PNPG at concentrations ranging from 0.5-25 mM.. Values for K_m and V_{max} were determined from Lineweaver-Burk plots.

2.11 Determination of inhibition constants

Inhibition by glucose of β-glucosidase was determined in the presence of PNPG as the substrate. Inhibition constants (K_i) were determined from corresponding Lineweaver-Burk plots using standard linear regression techniques.

2.12 Internal amino acid sequence of β-glucosidase by LC-MS/MS

The internal amino acid sequence of β-glucosidase was performed by in-gel digestion of the protein and sequencing of the different peptides by mass spectrometry. The ion spectra were analyzed and the sequence determined. The analysis was performed at the National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand. Sequence comparison of internal peptides of β-glucosidase with those of the β-glucosidase family was performed using individual peptides. Peptide matching from sample mass spectra was based on an accuracy of ± 1 Da. Peptide. Amino acids were matched to the SWISSPROT data.

3. Results and discussion

3.1. Culture conditions and production of β-glucosidase activity

Carboxy-methylcellulose was the most effective inducer of β-glucosidase activity of the carbon sources tested. Microcrystalline cellulose (Avicel® PH-101) and filter paper were also fairly good inducers but with glucose as a carbon source enzyme production was repressed. The highest level of total β-glucosidase activity (8.102 U/ml) was produced in carboxymethyl-cellulose (CMC) after 10 days growth.

3.2 Purification of β-glucosidase

β-glucosidase was successfully purified through ammonium sulfate precipitation, SP Sepharose, Phenyl Sepharose, and Superdex-200 column chromatography (Table 1). The β-glucosidase was purified 50.23 fold with 6.28% retention of total extracellular activity and 0.13% retention of total protein. The specific activity of the purified enzyme was 77.86 U/mg of protein. The specific activities of purified β-glucosidases from various microorganisms examined by other researchers varied from 5 to 979 U/mg of protein [16-20].

Table 1. Purification table of β-glucosidase from *D. eschscholzii*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	695.47	1078.96	1.55	100.00	1.00
Ammonium sulfate	41.63	343.42	8.25	31.83	5.32
SP Sepharose (ion exchange)	3.75	175.61	46.83	16.28	30.21
Phenyl Sepharose (hydrophobic interaction)	1.52	98.53	64.72	9.13	41.75
Superdex-200 (gel filtration)	0.87	67.74	77.86	6.28	50.23

3.3 Molecular weight and isoelectric point determination

SDS-PAGE analysis of the purified β-glucosidase showed the presence of a single band when stained with Coomassie Blue R-250 and its apparent molecular mass was about 64.2 kDa (Figure 1A), indicating that the enzyme is a monomer. The molecular mass of β-glucosidases from aerobic fungi range from 40 to 480 kDa [21, 22]. Analytical IEF data demonstrated that β-glucosidase from D. eschscholzii is an alkaline protein; it was isoelectric at pH 8.55 (Figure 1B). β-Glucosidases from aerobic fungi generally have acidic *pIs* [1]. However, β-glucosidases from fungi with basic pIs have also been reported, e.g. Trichoderma reesei [23, 24].

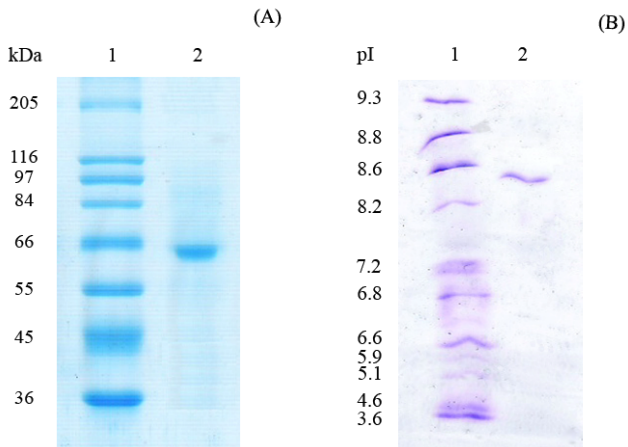

Figure 1. (A) SDS-PAGE of purified β-glucosidase from *D. eschscholzii*: lane 1, molecular weight marker of protein standard, i.e., myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehydes-3-phosphate dehydrogenase (36 kDa); lane 2 purified β-glucosidase (10 µg of protein). (B) IEF-PAGE of purified β-glucosidase from *D. eschscholzii*: lane 1, *pI* marker i.e., amyloglucosidase from aspergillus niger (3.6), trypsin inhibitor (4.6), β-lactoglobulin A (5.1), carbonic anhydrase II (5.9), carbonic anhydrase I (6.6), myoglobin (6.8, 7.2), lentil lectin (8.2, 8.6, 8.8), trypsinogen from bovine pancreas (9.3); lane 2, purified β-glucosidase (5 µg of protein).

3.4 Effect of temperature and pH on β-glucosidase activity and stability

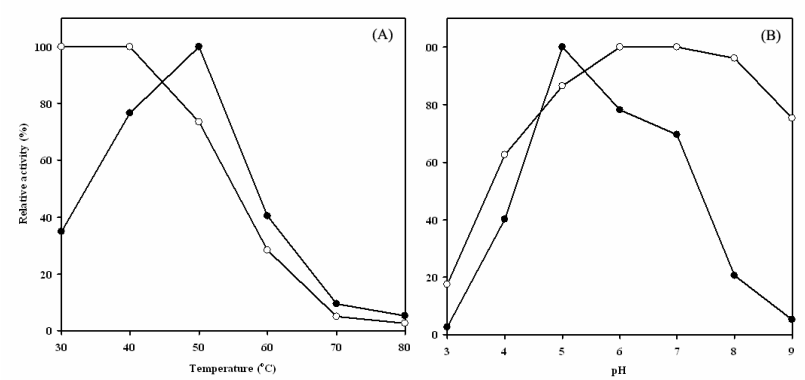

Figure 2. (A) Temperature activity (●); and temperature stability (○). The following buffer systems were used 0.1 M acetate pH 5.5. (B) pH activity (●); and pH stability (○). The following buffer systems were used: 0.1 M sodium acetate buffer (pH 3.0-6.0); 0.1 M phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.0-9.0). The values shown represent averages from triplicate experiments.

β-Glucosidase from *D. eschscholzii* displayed maximal activity at 50 °C (Figure 2A). Similar temperature optima of β-glucosidases ranging from 50 to 65 °C have been reported from several fungi, such as *Aspergillus niger* [25], *Fusarium oxysporum* [26], and *Neurospora crassa* [27]. Thermostability of the enzyme at different temperatures was monitored and the enzyme was found to be fairly stable at temperatures up to 60 °C for 30 min. It was completely inactivated on incubation at 70 °C for 30 min (Figure 2A). The pH optimum for β-glucosidase activity was 5.0 (Figure 2B) which is similar to that reported for the β-glucosidase from *Aspergillus* species [25, 28]. The enzyme was fairly stable over the pH range of 5.0 to 8.0, retaining over 85% activity. The enzyme, however, was shown to be sensitive to pH below 4.0 since it lost its activity at pH 3.0. Conversely, it was found to be very stable under neutral and alkaline pH since it retained up to 75% of its activity at pH 9.0 (Figure 2B). Most fungal β-glucosidases exhibit pH optima ranging from 5.0 to 6.5 [29].

3.5 Effect of metals and reagents

The enzyme activity was strongly inhibited by the sulfhydryl oxidant which has been generally reported as a strong inhibitor for β-glucosidases but it was observed that some metal ions activate the enzyme (Table 2). This result suggested that the thiol group was essential for β-glucosidase activity [30]. This was further confirmed by the observation that Hg^{2+} ions completely inactivated the enzyme. The chelating agent EDTA did not affect β-glucosidase activity indicating that β-glucosidase is not a metalloprotein. Furthermore, DTT is not an inhibitor suggesting that disulfide bonds are not essential for enzyme activity. Activation by Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, glycerol and DMSO may be explained by stabilization of the enzyme structure.

Table 2. Effect of some cations and other reagents on purified β-glucosidase activity from *D. eschscholzii*

Reagent[a]	Relative activity (%)[b]
Control[c]	100.0
$CaCl_2$	107.3
$CoCl_2$	101.6
$FeCl_2$	78.7
$HgCl_2$	6.8
$CuSO_4$	85.2
$MgSO_4$	122.3
$MnCl_2$	118.5
$ZnSO_4$	92.0
Glycerol	104.6
DMSO[d]	102.8
DTT[e]	107.5
EDTA[f]	108.3

[a]The assays were carries out in the presence of 1 mM concentrations of each of the metal ions and reagents.

[b]The relative activity was determined by measuring β-glucosidase at 50 °C in 0.1 M sodium acetate buffer pH 5.0 after pre-incubation at 30 °C for 30 min with individual cations or reagents. Results are shown as the average p-nitrophenol released from a representative assay performed in triplicate.

[c]The activity assayed in the absence of cations or reagents was taken as 100%.

[d]DMSO; dimethyl sulfoxide

[e]DTT; dithiothreitol

[f]EDTA; ethylenediaminetetraacetic acid

3.6 Substrate specificity

The purified β-glucosidase from *D. eschscholzii* is a broad-specificity type since it can hydrolyze a range of (β-1, 2), (β-1, 3), (β-1, 4), and (β-1, 6) diglycosides, as well as saccharides and aryl-β-glycosides. The purified enzyme had none or very little (≤8.5%) activity on lactose, maltose, sucrose, Avicel, and carboxymethyl cellulose. It had very little (≤7.0%) activity on *p*-nitrophenyl-β-D-xylopyranoside and *o*-nitrophenyl-β-D-galactopyranoside. *o*-Nitrophenyl-β-D-glucopyranoside was hydrolyzed at 15.5% of the level of hydrolysis of PNPG (Table 3). β-Glucosidases with very broad specificity have been isolated from many fungi [31, 32].

Table 3. Relative initial rates of various substrates by purified β-glucosidase activity from *D. eschscholzii*

Substrate	Relative activity (%)[a]
Saccharides	
cellobiose (20 mM) (β-1, 4) Glc	100.0
sophorose (20 mM) (β-1, 2) Glc	79.5
laminaribiose (20 mM) (β-1, 3) Glc	65.7
gentiobiose (20 mM) (β-1, 6) Glc	76.8
lactose (20 mM) (β-1, 4) Gal	2.5
maltose (20 mM) (α-1, 4) Glc	6.2
sucrose (20 mM) Frc (α-1, 2) Glc	0.0
Avicel® (1%, wt/vol)	0.0
carboxymethyl cellulose (CMC; 1%, wt/vol)	8.5
Aryl-glycosides	
p-nitrophenyl-β-D-glucopyranoside (5 mM)	100.0
o-nitrophenyl-β-D-glucopyranoside (5 mM)	15.5
p-nitrophenyl-β-D-xylopyranoside (5 mM)	7.0
o-nitrophenyl-β-D-galactopyranoside (5 mM)	0.0

[a]Values shown are the averages from triplicate experiments with each substrate. Activity of the saccharides was determined by measuring the release of glucose (DNS method), and on aryl-glucosides by measuring the release of PNPG. Sufficient enzyme was used to ensure a linear release of product during the first 10 min of reaction at 0.1 M sodium acetate buffer pH 5.0 and 50 °C. The relative initial rate of hydrolysis of a saccharide is expressed as a percentage of that obtained with cellobiose and that of an aryl-glucoside is expressed as a percentage of that obtained with PNPG.

3.7 Determination of kinetic parameters and inhibition constants

Reaction kinetics of the purified β-glucosidase were determined from Lineweaver-Burk plots with PNPG as substrate under defined assay conditions. The enzyme had K_m values of 1.52 mM, and V_{max}, values of 3.21 U/min/mg of protein (Figure 3A). In the case of β-glucosidase from *D. eschscholzii*, the K_m values for PNPG are similar to those of other fungal β-glucosidases such as from *Aspergillus fumigatus* [33], *Aspergillus wentii* [34], and *Sclerotium rolfsii* [35] with K_m values of 1.4, 1.6, and 1.38 mM respectively. Glucose was found to be a competitive inhibitor of the enzyme as shown by a Lineweaver-Burk plot in the presence of various concentrations of glucose. The K_i was found to be 0.79 mM for glucose when PNPG was used as the substrate (Figure 3B). Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases although there are exceptions like β-glucosidases produced by several *Aspergillus* species [25, 28, and 36]. Most microbial β-glucosidases have glucose inhibition constants (K_i) ranging from 0.35 mM to no more than 100 mM [28]. The glucose inhibition constants for β-glucosidases are the smallest reported for fungal β-glucosidases [26, 31].

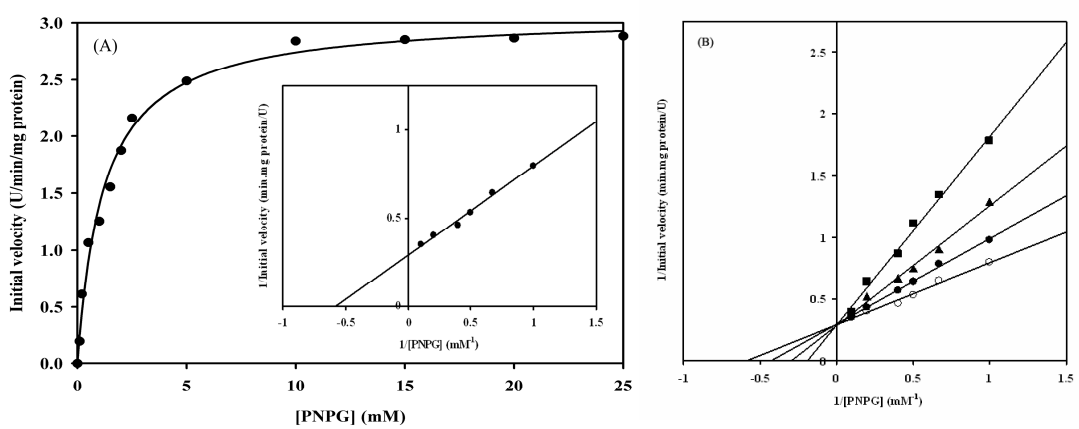

Figure 3. (A) Variation of initial velocity with PNPG concentration in the hydrolysis reaction of β-glucosidase from *D. eschscholzii*. K_m and V_{max} were 1.52 mM, and 3.21 U/min/mg of protein, respectively. (B) Lineweaver-Burk plot of PNPG hydrolysis by purified β-glucosidase in the presence of glucose. Addition of glucose: (○) 0 mM; (●) 2.5 mM; (▲) 5.0 mM; and (■) 10 mM. The intersection with the abscissa yielded a K_i and was found to be 0.79 mM.

3.8 Internal amino acid sequence of β-glucosidase by LC-MS/MS

The internal sequence analysis of the purified β-glucosidase was obtained by digestion with trypsin and sequence analysis with LC-MS/MS and was found to be TDGSNGDVASDWYHR. Comparisons were then made to all protein sequences in the SwissProt database using the search protocol BLAST. A high degree of internal amino acid sequence identity between *D. eschscholzii* β-glucosidase and other β-glucosidases of family 3 suggested that this enzyme could be a member of glycoside hydrolase family 3 (Figure 4). In addition, the active site of this enzyme contains several conserved residues, including a nucleophile (Asp) and an acid/base catalyst (Glu). Sequence alignment of the region containing the proposed catalytic nucleophile of over 15 β-glucosidases of family 3 shows the presence of Asp as a fully conserved catalytic nucleophile [37]. The data presented here showed that the β-glucosidase from *D. eschscholzii* follows a retention mechanism and most probably belongs to family 3 of the glycoside hydrolase, as well as possessing an Asp or a Glu residue as a catalytic nucleophile.

Daldinia eschschozii		I	T	D	G	S	N	G	D	V	A	S	D	W	Y	H	R	
Aspergillus fumigatus	280	A	E	L	G	F	Q	G	F	V	M	S	D	W	S	A	H	295
Aspergillus niger	269	A	E	L	G	F	Q	G	F	V	M	S	D	W	A	A	H	284
Fusarium graminearum	284		E	M	G	F	Q	G	F	V	M	S	D	W	Q	A	Q	298
Neurospora crassa	278		E	L	G	F	Q	G	F	V	M	S	D	W	L	S	H	292
Phanerochaete chrysosporium	163	I	A	D	G	S	S	G	D	V	A	T	D	S	Y	N	R	210
Talaromyces emersonii	272			D	G	T	N	G	D	V	A	C	D	S	Y	H	R	313
Trichoderma reesei	139		T	N	G	A	N	G	D	V	A	C	D	H	Y	H	A	183

Figure 4. Amino acid sequences from the fragment obtained by tryptic digestion of the purified β-glucosidase from *D. eschscholzii*. Comparisons are made with other β-glucosidases classified as family 3 glucosyl hydrolases. Shaded regions represent regions of identity.

Acknowledgements This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0105/2546). We wish to Thanks Dr. Surang Thienhirun for providing the specimen from Royal Forest Department, Bangkok, Thailand.We would like to express our sincere thanks to Professor G.M. Gadd for his helpful advice in reviewing this manuscript.

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Relationships between light-treated cultures and lactose content in yogurt

M. Stijepic[1], M. Solaja[2], D. Durdevic Milosevic[*,3], and Lj. Topalic Trivunovic[2]

[1] Sanitary Engineering Department, High Medical School, Nikole Pasica 4a, 79 000 Prijedor, Republic of Srpska, Bosnia and Herzegovina

[2] Department of Food Technology, Faculty of Technology, Stepe Stepanovica 73a, 78 000 Banja Luka, Republic of Srpska, Bosnia and Herzegovina

[3] Department of Food Technology, Higher Technological School of Professional Study, Hajduk Veljkova 10, 15 000 Sabac, Republic of Serbia

[*]Corresponding author: E-mail: dadadada@panet.co.yu , Phone: +387 64 416 92 29

The abilities of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus to decrease lactose content in milk after different light treatments have been compared in this study. Each one of cultures was treated by visible daily light, linear non-coherent polarized light or kept in dark. After fermentation lactose concentration was measured by ultrasound milk analyzer Lacto Scan. Experimental results show the decrease of lactose concentration in a range of 37,5% - 40,35%, dependant to the treatments and durations. There are significant differences in lactose concentration ($p < 0,01$) by cultures treated 40, 50 and 60 minutes, in relation to the kind of treatments. Each one of treatments in duration 30 show no statistical differences in lactose concentration ($p = 0,05$). Diagram of experimental results presents a percent of decrease in lactose concentration as a logarithmic function of the energy impute in the case of linear non-coherent polarized light.

Keywords Lactobacillus delbrueckii subsp. bulgaricus; lactose; light; Streptococcus termophilus; yogurt

1. Introduction

Lactose or milk sugar (4-O-b-D-galactopyranosyl-D-glucose) is a disaccharide sugar composed of glucose and galactose. Lactose is found in milk and milk products. It is unique to mammalian milks and first dietary sugar to which newborns are exposed.

Lactose in milk can be fermented by lactic acid bacteria to lactic acid which causes the characteristic curd to form. The starter culture for most yogurt production is a symbiotic blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subp. *Bulgaricus* [1]. The lactic acid bacteria used in the production of yogurt accomplish two tasks: production of lactic acid and flavor components. *Streptococcus thermophilus* grows faster and produces both acid and carbon dioxide. The formate and carbon dioxide produced stimulates *Lactobacillus delbrueckii* subp. *bulgaricus* growth. Yogurt is defined by the Codex Alimentarius of 1992 as a coagulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [2]. These microorganisms are responsible for the decrease of lactose in yogurt. One example of a significant bacteria-induced change that occurs during the fermentation process is the hydrolysis of 20-30 % of the disaccharide lactose to its absorbable monosaccharide components, glucose and galactose [2].

Lactose in yogurt is better digested than lactose in other dairy foods by lactase-deficient individuals [3, 4, 5, 6, 7]. Many adults have trouble digesting lactose and dairy products due to low levels of lactase enzyme needed to break down lactose in intestine [8, 9, 10]. Yogurt fermentation produced growth benefit as compared to the milk from which it was made by reducing the lactose content and by supplying microbial lactase activity [11, 12, 13, 14, 15]. Yogurt bacteria have been shown to stimulate the gut immune system [16, 17] and also the immunological and genetic basis of the immunostimulatory properties of yogurt starters has been investigated [18, 19, 20].

Lactose content was elevated in yogurt of cow milk, with a mean and standard deviation of 3.81 ± 0.47 gm% [21]. Large quantities of yogurt are consumed by some lactase-deficient population groups. Some of directions in a production of milk fermented products are productions of low lactose products [22, 23, 24]. The aim of this work was to investigate influence of different light treatments of lactic acid bacteria in a sense of decreasing lactose content during the milk fermentation.

Yogurt bacteria were found in human feces, suggesting that they can survive transit in the gastrointestinal tract [3, 25] and also dark condition.

The results reported in many papers indicate different effects of natural and artificial light upon microbial growth and metabolic products [26, 27, 28, 29, 30]. Some of them are germicidal actions in UV region of spectrum, the recovery in dark condition, mutagenic effects, and change in metabolic pathways.

2. Material and methods

In this study we compared the abilities of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* to decrease lactose content in milk after different light treatments. Each one of culture was treated by visible light, non-coherent polarized light or kept in dark. Source of visible non-coherent polarized light was a lamp Bioptron Compact III (Zepter, Switzerland) with technical characteristics of light as follows: wavelength 400-2000 nm, linear polarization >95 % and constant radiation dose 2.4 J/cm^2 per minute.

Constant radiation dose 2.4 J/cm^2 per minute and duration of treatments (30, 40, 50 or 60 minutes) were used in calculation of energy impute (J/cm^2) of linear non-cohernet polarized light. For inoculation were used *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptocuccus thermophilus* as starter culture Yoghurt V1 Visbyvac Dip 5u (Danisco Cultor, Niebull, Germany).

Samples of 2.8 % fat pasteurized milk were inoculated by culture (5 g culture per 1 liter milk) and incubated at 42 ^{o}C for 5.5 hours. The cultures were treated by visible light, non-coherent polarized light or kept in dark before the inoculation. The treatments' duration was 30, 40, 50 or 60 minutes.

Lactose content was measured by ultra sound analyzer LactoScan (Micotronic LTD, Made in Europe) in 60 samples of milk and 180 samples of fresh made fermented products. ANOVA was used for calculations and analysis.

3. Results and disscusion

A mean and standard deviation of lactose content in samples of milk (n=20) was 4,558 ± 0,00086 %, 4,582 ± 0,000289 %, 4,578 ± 0,000474 % and 4,578 ± 0,000387 % before the treatments of 30, 40, 50 and 60 minutes, respectively.

The ranges of lactose content in 180 samples of yogurt after different treatments of culture are presented in Table 1.

Results of statistical analysis show no significant difference in lactose content after different treatments in duration 30 minutes (p=0,05). The statistical analysis show but significant difference in lactose content between different treatments in duration 40 minutes (p<0,01), in duration 50 minutes (p<0,01), and in duration 60 minutes (p<0,01).

There are significant differences in lactose content, between 30 and 40 minutes treatments (p<0,01), and also between 30 and 60 minutes treatments (p<0,01).

Table 1. Lactose content in yogurt after different treatments of culture

Duration of treatments (min)	The range of lactose content in yogurt after treatment (%)		
	Non-coherent polarized light	Dark	Visible light
30	2.49-2.99	2.55-2.91	2.58-2.81
40	2.84-2.90	2.85-2.88	2,84-2,87
50	2.85-2.89	2.80-2.85	2.83-2.87
60	2.84-2.86	2.82-2.85	2,80-2,84

The averages of lactose decrease (%) after different light treatments of culture are presented in Table 2.

Table 2. The average of lactose decrease (%) after different light treatments of cultures

Duration of treatments (min)	The average of lactose decrease (%)		
	Non-coherent polarized light	Dark	Visible light
30	39,18	40,15	40,35
40	37,25	37,54	37,62
50	37,33	38,25	37,81
60	37,83	38,03	38,49

The lowest decrease of lactose was reached in case of 30 minutes treatment of linear non-coherent polarized light. On the other side, for the same time (30 minutes) but by visible light treatment lactose degrease has had the biggest value.

Figure 1. shows the diagram how non-coherent polarized light influences the decrease of lactose concentration.

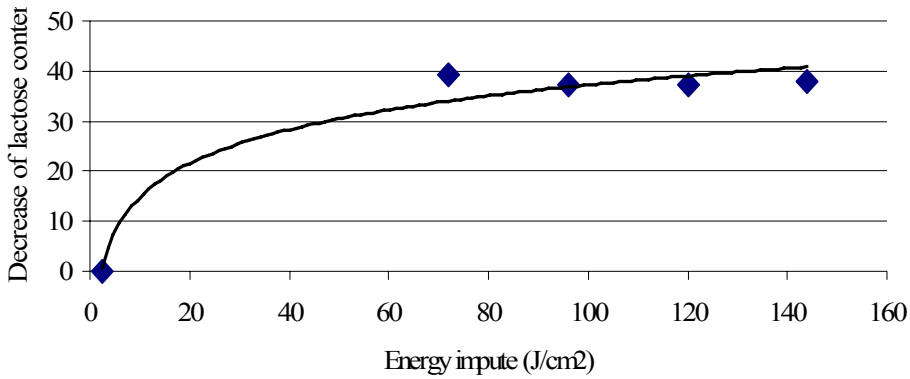

Figure 1. Diagram of energy impute of non-coherent polarized light influences upon decrease of lactose content in yogurt

The trend-line shows the logarithmic function presented in Eq. (1) with very high coefficient of determination $R^2 = 0.9661$.

$$D = 9.7659\ln(E) - 7.7718 \quad (1)$$

where is:
D – decrease of lactose content (%)
E – energy impute of linear non-coherent polarized light

4. Conclusion

This study compared the abilities of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus termophilus* to decrease lactose content in milk after different light treatments.
Experimental results show decrease of lactose concentration in range 37,5 - 40,35 % depending of the kind of treatment and treatment's duration. Low-lactose milk products with 70 % or more of the lactose hydrolysed by food grade β-galactosidase enyzmes of yeasts or fungi have become widely accepted for alleviating the symptoms of lactose maldigestion [31].

There are significant differences in lactose content between different treatments of culture in duration 40, 50 and 60 minutes ($p < 0,01$). Each one of treatments in duration 30 shows no significant difference in lactose content ($p = 0,05$). There are significant differences in lactose content between 30 and 40 minutes treatments, 30 and 50 minutes treatments, and 30 and 60 minutes treatments ($p<0,01$).

Diagram of experimental results presents a percent of decreasing lactose concentration as a logarithmic function of the irradiation energy impute in the case of incoherent polarized light.
Our results show the possibility of light treatments' manipulation of starter culture in a sense of decrease lactose content in yogurt.

Subsequent treatments in durations lower than 30 minutes could prove to be very interesting too.

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The use of $ScCO_2$ for the extraction of LPS from *S. enterica subsp.* PCM 2266

J.Rybka[a], P.Grycko[ac], J. da Cruz Francisco[c], A.Gamian[a,b] and E. S. Dey[c,*]

[a] Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wroclaw, Poland

[b] Department of Medical Biochemistry, Wrocław Medical University, Chałubińskiego 10, 50-368 Wrocław, Poland

[c] Lund University, Pure and Applied Biochemistry, 221 00 Lund, Box 124, 212 00 Lund, Sweden

[*]Corresponding author: Tel:+46 46 222 8258; Fax:+46 46 222 4611; E-mail: estera.dey@tbiokem.lth.se

The paper describes a novel extraction procedure for lipopolysaccharides (LPS) from *Salmonella enterica* subsp. *enterica* (PCM 2266). Process parameters for the extraction of LPS from bacterial mass were optimized by carrying out a two-level fractional design experiment. Four parameters, namely temperature, CO_2 flow rate, pressure and co-solvent composition were analyzed. The best crude extract yields were achieved when the CO_2 flow rate and temperature were kept high (10g/min, 90°C) and pure water was used as a co-solvent. Pressure had no statistically significant effects within the range of the study performed, whereas the other factors were relevant. The recovery of the extracted LPS by $scCO_2$ was about 3.3% of the biomass used, while in the classical extraction method yield was less than 2%. All isolates were characterized by SDS-PAGE, by the spectra of the thiobarbituric acid reaction products and GLC-MS analysis.

Keywords lipopolysaccharides (LPS); Supercritical carbon dioxide ($scCO_2$); Salmonella, vaccine

1. Introduction

Lipopolysaccharides (LPS) also known as endotoxins, are an integral part and the most abundant components of the outer membrane of Gram-negative bacteria. The amphiphilic LPS molecules consist of hydrophilic polysaccharide and hydrophobic lipid moieties. Three main regions distinguished in LPS molecule are: lipid A, core oligosaccharide and O-specific polysaccharide. The lipid A is linked by the ketosidic linkage of the 3-deoxy-D-manno-octulosonic acid (Kdo) residue to the saccharide part of LPS. The Kdo residue can have different substituents some of the substituents are acid labile [1, 2].

To investigate the biological activity of LPSes, they are isolated by classical methods using organic solvents eg., mixture of hot phenol-water (PW) [3], or phenol-chloroform-petroleum ether (PCP) [4]. The lesser known procedures employ mechanical disruption of bacterial cells, followed by the enzyme treatment and chelating agents [5] 6] [7]. All the methods are useful in a laboratory scale, laborious and give low yields.

The methods that are using organic solvents create toxic wastes and contamination of the final product with solvent residues. Currently, there is an increasing demand on the market for LPS preparations, free from toxic impurities if used as vaccines. Supercritical carbon dioxide ($ScCO_2$) extraction is a safe alternative to solvent flammable and hazardous organic solvents. It is relatively simple to modify $scCO_2$ solvent properties like density, by pressure and temperature change and polarity by addition of small amount of selective co-solvents. The extraction can be adapted to the specific needs of the user, e.g. for the extraction of more hydrophobic or more polar substances.

The paper describes a new method for the extraction of a model LPS, using $scCO_2$ combined with water.

2. Materials and methods

2.1 Chemicals

The ammonium salt of 2-keto-3-deoxy-octulosonic acid (Kdo) was used in our previous work [8]. DNA oligonucleotides (24 bp) were from Fermentas Sweden, Life Science, Follin-Ciocalteu's phenol reagent, bromophenol blue, thiobarbituric acid (TBA) and lipopolysaccharide (LPS) from *Salmonella thyphimurium* were from Sigma (St Louis, US). All other chemicals were of analytical purity. The carbon dioxide (≥99.998%) used for the extraction was from AGA Gas, Sundbyberg, Sweden.

2.2 Bacterial strains and growth conditions

Strains of *Salmonella enterica* subsp. *enterica* (Re - PCM 2266, Rc – PCM 2263 and Ra – PCM 2260) were obtained from the Polish Collection of Microorganisms (PCM), Institute of Immunology and Experimental Therapy Wroclaw, Poland. Bacteria were grown from a 200 ml inoculum (E_{600}=0.8) in 10 l flasks filled with LB media. Culture was agitated (150 rpm) at 37° C for 24 h, harvested by centrifugation (6000 x g, 20 min, 4 ºC), and washed twice with phosphate-buffered saline, frozen down in liquid nitrogen and freeze dried in the equipment from Ninolab AB (Sweden).

2.3 Extraction of LPS with phenol - chloroform - petroleum ether mixture (PCP)

PCP extraction [4] was made as follows: 400 mg of freeze-dried biomass was extracted with 3 ml of PCP mixture composed of 90% phenol : chloroform : petroleum ether, (4 : 10 : 16 w/v), respectively which was added and vigorously shaken at 37 ºC for 5 min. and centrifuged in Eppendorf centrifuge at 3000 rpm at room temperature for 15 min. The pellet was re-extracted with a new portion of PCP and centrifuged. LPS was precipitated by drop-wise addition of water to the combined supernatants. The precipitate was collected by centrifugation in Eppendorf centrifuge at 3000 rpm for 20 min and washed twice with 80% phenol, three times with acetone and finally freeze-dried.

2.4 Extraction of the LPS with $scCO_2$

2.4.1 Sample pre-treatment

200 mg of freeze-dried biomass was soaked in 2 ml (30mM) sodium triphosphate pentabasic (STPP), a chelating and protein binding agent [9] and incubated at room temperature for 1h to permeabilize the cell membranes and liberate protein free LPS.

2.4.2 Extraction process with scCO2

A SFE-2X100F extraction system, Thar Technology Inc. Pittsburgh (PA, USA) was used [10]. Pre-treated biomass (2.4.1) between two filter paper layers was placed in the extraction vessel (50ml) filled with the glass wool. The extractions were performed in duplicates. Collected extracts were freeze-dried and further analyzed.

2.4.3 Detection of LPS

The LPS was quantified by a colorimetric test with thiobarbituric acid (TBA) [11], gas-liquid chromatography connected with mass spectrometry (GLC-MS) [8] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 5% stacking and 20% resolving (Sigma-Aldrich).

3. Results and discussion

3.1 Extraction of LPS by classical phenol-chloroform-petroleum ether (PCP) method

A PCP extraction was carried out to prepare a reference material to compare with the currently developed $scCO_2$ extraction method. The recovery of LPS from $scCO_2$ is almost twice higher than the recovery from the PCP method (Table 1).

It is the first time; a pioneering scCO2 extraction method has been successfully tested for the isolation of rough-type bacterial LPS from *Salmonella enterica* subsp. *enterica* PCM 2266.

Procedure	Freeze-dried biomass [mg]	LPS [mg]	LPS recovery [%]
$scCO_2$ [0-6h]	200	6.72	3.36
PCP method	400	7.4	1.86

Table 1. LPS extracted by $scCO_2$ compared to PCP extracts. The $scCO_2$ material was from the experiment shown in Figure 3.

3.2 Extraction of LPS by $scCO_2$ /Ruggedness Test

The extraction of LPS from bacterial biomass is highly dependent on the polarity of the used co-solvent. The polarity of the co-solvent was increased by the addition of 15% mixture of water: methanol (9: 1) and/or pure water (Fig. 1).

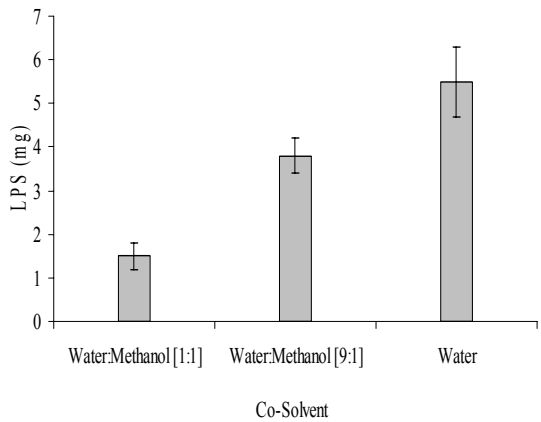

Fig.1. Testing the co-solvent composition under the following extraction conditions: extraction time – 1.5h; $scCO_2$ flow – 10g/min; temperature – 90°C; pressure – 250 bar; and 15% co-solvent.

Before setting up the factorial design, the factors such as temperature and extraction time were tested for the optimal extraction of LPS. The tested temperatures were 60, 75, 85 and 90°C and extraction times 1-2.5 h at 30 minutes intervals were tested at constant pressure (250 bar) and 15% mixture of water: methanol (9:1) as co-solvent (Fig. 2).

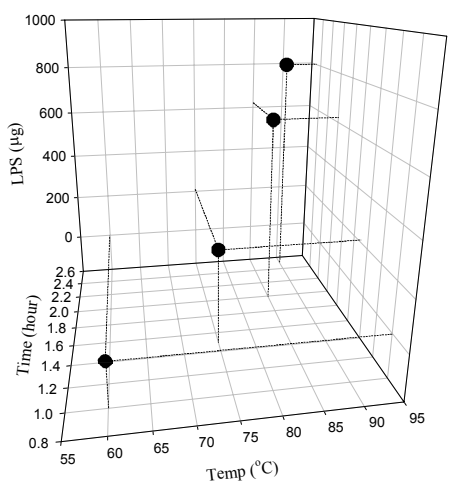

Fig.2. LPS release from bacterial cells as a function of time and temperature. $ScCO_2$ flow – 10g/min; pressure – 250 bars; and 15% co-solvent – water: methanol (9:1) was constant. All points are means from duplicate tests.

Higher temperature and extended extraction time (up to 2.5 h) resulted in the highest amount of LPS. In the ruggedness test 1.5 h was used as extraction time, where four factors: temperature, CO_2 flow, pressure and type of co-solvent, were analyzed at two levels by the ruggedness testing method (Table 2).

We could also include extraction time as the fifth factor. Since the range of the factor was unclear and more systematic study was later made (Fig.3), we kept it constant for the test. The temperature range was set, based on results shown in Fig. 2. As co-solvent on the low level in the ruggedness test, pure water was used. The combinations of parameters tested and amount of extracted LPS are shown in the Table 2. Table 3 summarizes the main effects, with confidence limits at 95% and confidence intervals of means [12].

Test No	Flow g/min	Press. bar	Temp. C	Co-Sol.
1	-	-	-	-
2	+	+	-	-
3	+	+	+	+
4	-	-	+	+
5	-	+	+	-
6	+	-	-	+
7	-	+	-	+
8	+	-	+	-

Table 2. Chart of Ruggedness Test. Factor analyzed; High level (+):10g/min CO_2, 250 bar, 90°C, 15% co-solvent: [H_2O:Methanol (9:1)] Low level (-): 5g/min CO_2, 200 bar, 60°C, 15% co-solvent: [H_2O], Extraction time 1.5h.

Factor analyzed	Main effect	Confidence limit	Confidence interval
Flow [g/min]	761.1	±146.8	614.2 to 907.9
Pressure[bar]	-51.1		-198.0 to 95.6
Temperature[C]	470.2		323.4 to 617.1
Co-solvent	-29.8		-176.7 to 116.9

Table 3. Results of the Ruggedness Test.

The ruggedness test indicates that temperature and CO_2 flow rate are the factors with the greatest influence on LPS extraction. The important role of the temperature in the extraction process tallies with the previous observation (Fig. 2). Increased pressure to 250 bars did not have significant effect on the extractability of LPS, and the presence of methanol in the co-solvent has a negative effect. The principle of ruggedness test for the calculation of the main effect of a factor is a simple computation where from sum of totals at the high level (+) minus the sum of totals at low level (-) of the factor. The resulting difference is divided by 8, since there were eight test results used in each of the two sums [13]. From the pattern of extractability, one can see that the amount of the extracted LPS is still high up to 3 h (Fig. 3).

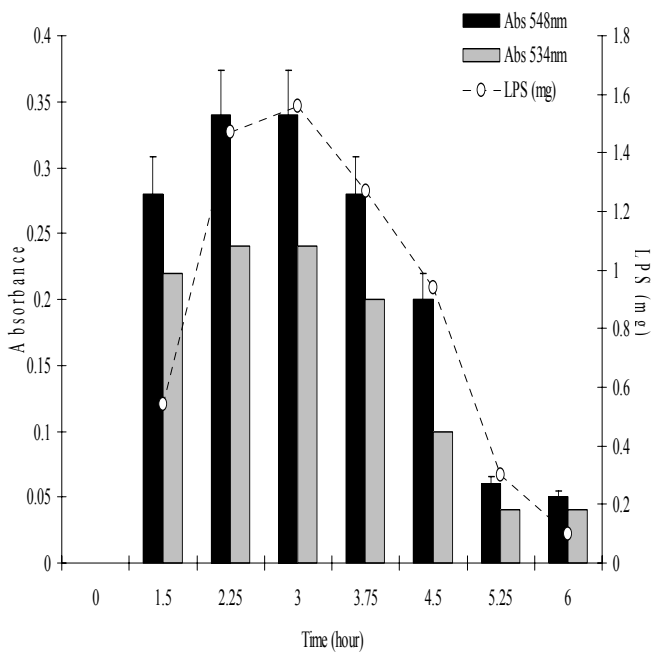

Fig.3. Prolonged extraction of LPS monitored with TBA test. Extraction conditions were: flow of CO_2 – 10g/min; co-solvent – 15% water; pressure – 200bar; temperature – 90°C; time – 6 hours.

After 3 h, a total 3.8 mg of LPS was recovered, i.e. almost 2% of the biomass used, while 6h extraction resulted in 6.7 mg of extracted LPS (Fig. 3) 3.3% of the biomass, Table 1. The comparison of absorbance ratio at A_{548}/A_{534} in the TBA test of the preparation collected indicates that the proportion between LPS and the

contaminating, deoxysugars-containing material changes over time with the best purity in the time range 1.5 – 3.0 h.

Fig. 4 shows the SDS-PAGE separation pattern of various LPS preparation developed by silver staining.

Lanes 2 - 6 show LPS preparations from rough mutants of *Salmonella enterica* subsp. *enterica* with various lengths of core oligosaccharides. In lanes 3, 4 and 6 the LPS extracts are from the same strain (2266), isolated by PCP (lane 3) and $scCO_2$ (lane 4 and 6), showing the highest mobility. The lane 4 shows dark smearing impurities that are not present in the corresponding isolate obtained using PCP (lane 3) and less smearing sample was received after 2.25 h of $scCO_2$ extraction (Fig. 4, lane 6).

Presented results can lead directly to the commercial and therapeutic applications in the forthcoming future [14].

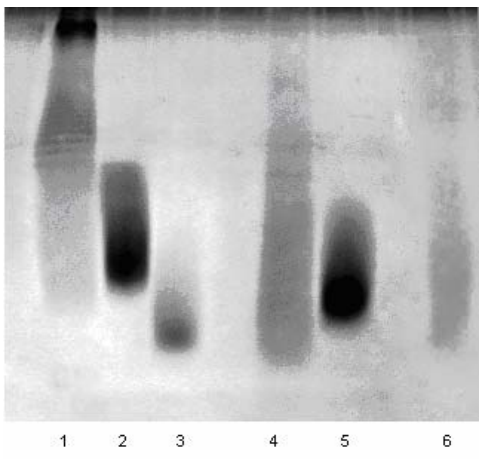

Fig.4. SDS-PAGE analysis of LPS extracted by $scCO_2$ compared to PCP extracts from *Salmonella* strains. Lane 1, (6µg) smooth LPS from *S. thyphimurium* (Sigma, L6511); Lane 2, (0.5 µg) PCP extract from *S. enterica* subsp. enterica Ra PCM 2260; Lane 3, (0.5µg) PCP extract from *S. enterica* subsp. e*nterica* Re PCM 2266; Lane 4, (15µg) $scCO_2$ extract from *S. enterica* subsp. e*nterica* Re PCM 2266 (1.5 h, Fig.3); Lane 5, (0.5µg) PCP extract from Rc PCM 2263; Lane 6, (15µg) $scCO_2$ extract from *S. enterica* subsp. e*nterica* Re PCM 2266 (2.25 h, Fig.3).

Acknowledgements This study was supported from the Project and Network Co-operation Visby Programme University Co-operation with Central Eastern Europe, Sweden.

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Use The Solid Fermentation as a New and Alternative Way for Xylitol Bioproduction

D. Tresinari dos Santos[*,1], **B. Fouad Sarrouh**[1], **J. César dos Santos**[1], **V. Haber Pérez and S. Silverio da Silva**[1]

[1] Department of Biotechnology, Faculty of Engineeging of Lorena/University of São Paulo (USP), 12602-810, Lorena, SP, Brazil

[*]Corresponding author: e-mail: diego_tresinari@yahoo.com.br, Phone: +5512 31595146

The use of the biotechnological way to xylitol production can represent minors production costs, whereas, in this case, it can be worked under reduced conditions of pressure and temperature and does not have necessity of purification of the hydrolysate. Among the searched alternatives, the Submerged Fermentation method is more extensively studied, having been tested, in recent years, several process options and operational conditions. However, little it has been studied on the use of Solid Fermentation method to this bioprocess. Therefore, considering the promising advantages of Solid Fermentation on the Submerged Fermentation in many biotechnological processes, this article has as objective to demonstrate the potentiality and applicability of the use of Solid Fermentation for xylitol production. For this, it has been done preliminary assays using sugarcane bagasse as inert support and xylose commercial as substrate in polypropylene sachets inside a greenhouse. The fermentation runs were done using the xylose fermenting yeast – C*andida guilliermnondii* at 30º C, under oxygen limited conditions.

Keywords Xylitol Bioproduction; Solid Fermentation; Inert support

1. Introduction

Xylitol is an alternative high added-value sweetener with anti-cariogenic properties of great concern for both the food industry and the biomedical sector. It is in fact largely utilized in the prevention and treatment of several pathologies, among which atopic dermatitis [1] pseudo-membranose colitis [2], medium acute otitis [3], osteoporosis [4], hemolytic anemia [5] and cystic fibrosis [6].

Nowadays, xylitol is chemically produced by catalytic hydrogenation of D-xylose. However, this process is too expensive owing to the need of using high pressure and temperature as well as several steps of raw material purification [7]. These reasons have stimulating the search of alternative methods for the production of such a polyol. To this purpose, the use of an optimized biotechnological process could be a cheaper way to produce xylitol, because it would take place under mild conditions and utilize not purified hemicellulosic hydrolyzates. However, the concentrations, yields and production rates obtained from fermentation media consisting in lignocellulosic hydrolyzates are still the bottlenecks of a large-scale process, although they can be improved by selecting the right fermentation system, operation mode and cultivation conditions [8].

Currently, the research works have been focused on the use of microorganisms, especially yeasts for xylitol production by Submerged Fermentation from hydrolysates. In this case, the biocatalyst is the microorganism, which, through complex systems multi enzymatic integrated, uses the xylose present in hemicellulosic hydrolyzates to obtain energy and, under certain conditions, allows the accumulation of polyalcohol in the fermentation medium.

The Solid Fermentation (SF) also called Fermentation in Solid State has been received especial attention when the issue is use of waste. In general the Solid Fermentation is a microbial process that develops on the surface of solid materials, which have absorbing property or of contain water, with or without soluble nutrients. These materials can be solid biodegradable or not. For this kind of fermentation is necessary that the microorganisms grow with dissolved nutrients under or on the liquid-solid interface [9].

The Solid Fermentation is characterized by 2 types: one, where the conditions for the solid state are offered by the substrate. In other SF, the development of the process occurs using an inert support, on it [10-11].

According to some old groups of researchers, they preferred the Submerged Fermentation, believing that the Submerged Fermentation was more convenient and more productive. In the last fifty years these groups grew more and more and SF was becoming forgotten. This decision was more or less arbitrary, because it weren't based on scientific knowledge dominated in the two cases, nor was done any kind of effective comparison. The reasons were probably the easier form to drive the Submerged process, its monitoring and control [12].

The SF presents several advantages due to its physical and chemical aspects, especially its reduced water activity and the formation of temperature gradients, nutrient and products. The SF differs significantly from

Submerged Fermentation, on the spore and production of enzymes and secondary metabolites, as well as the method of mixing and diffusion. The microscopic substrate heterogeneity of, once considered the weakness of the SF, is now regarded as its main force for the increase of products income due causing appropriate changes in microbial physiology. It is a process which favors itself the low water levels, generating an clean industrial process with low levels of residual water, which also incurs on energy economy in the process of recovery ("downstream") [9].

Therefore, considering the promising advantages of Solid Fermentation on the Submerged Fermentation in many biotechnological processes, this article has as objective to demonstrate the potentiality and applicability of the use of Solid Fermentation using sugarcane bagasse, an agro-industrial residue, as a inert support for xylitol production.

2. Materials and methods

2.1 Preparation of the inert support

The sugarcane bagasse was initially ground passing through a 14 mesh (1,41 mm) standard Tyler sieve and retained in a 35 mesh (0,5 mm) sieve. After separation and screening, the agro-industrial residue was washed with distilled water and dried at 100°C up to constant weight.

2.2 Determination of humid point

The humid point, the point in which we have the maximum amount of fermentation medium that the sugarcane bagasse can hold was estimated by the gradual addition of small quantities of distilled water in 1 g of support (dry weight) until its saturation.

2.3 Microorganism maintenance and inoculum preparation

Cells of the yeast *Candida guilliermondii* FTI 20037, belonging to the culture collection of the Department of Biotechnology of Faculty of Engineeging of Lorena of University of São Paulo, were maintained at 4°C in a medium containing agar malt extract (Merck, Darmstadt, Germany).

To prepare the inoculum, yeast cells were cultured at 30°C and 200 rpm for 24 h in 250 mL-Erlenmeyer flasks containing 50 mL of a cultivation medium composed of: xylose, 30 g/l; $(NH_4)_2SO_4$, 3 g/l; $CaCl_2.2H_2O$, 0.1 g/l and rice bran extract, 10% (w/v), placed in an incubator, model G25-KC (New Brunswick, Edison, NJ). After growth, the cells were recovered by centrifugation, model CU-500 (Damon/IEC, Needham, MA) at 2000 x g for 20 min, washed and resuspended in isotonic solution (de-ionized water) in order to get a highly concentrated cell suspension.

To obtain always the same inoculum (1.0 g/l wet weight), cell concentration in this suspension was determined by optical density measurements (see below) and then appropriate aliquots were added to the fermentation medium.

2.4 Fermentation conditions

All the fermentations were performed in polypropylene sachets inside a greenhouse, under a temperature of 30 °C, containing 7 g of sugarcane bagasse embedded with 70 mL of fermentation medium (proportion according the determination of humid point: 1g of sugarcane bagasse: 10mL of medium) with 1 g /l of cells. The fermentation medium was composed of: xylose, 50 g/l; $(NH_4)_2SO_4$, 3 g/l; $CaCl_2.2H_2O$, 0.1 g/l and rice bran extract, 10% (w/v).

One sachet was taken after regular time intervals of 24 h, for sampling analysis.

All the experiments were carried out in duplicate, and the standard deviations never exceeded 8%; therefore, no additional statistical analysis was considered to be necessary.

2.5 Solid-liquid extraction (“Leaching”)

After to take each sachet, its content was transferred for a ErlernMeyer of 250mL and added 50 mL of distilled water (solvent extractor), and followed by incubation in rotating movement greenhouse under 200rpm (G25 - KC, New Brunswick, Scientific Co) for 30 minutes at 30 º C (adapted from Sing et al., 1999). Subsequently, for the solid-liquid separation, it was made a vacuum filtration in the qualitative filter (14 μ m), and the filtrate analyzed by HPLC.

2.6 Analytical determinations

Cell concentration was determined by optical density (OD) measurements at 640 nm, using a spectrophotometer (model DU 640B, Beckman Coulter, Fullerton, CA). A previously constructed calibration curve was used to relate the OD measurements to dry cell concentration of samples of both this suspension as well as that used for inoculum.

Xylose and xylitol concentrations were measured by HPLC, model LC-10-AD (Shimadzu, Tokyo, Japan), equipped with an Aminex HPX-87H (300 x 7.8 mm) column (Bio-Rad, Hercules, CA) and a refractive index RID 6A detector. Samples were previously filtered through a Sep Pak C18 filter and injected in the chromatograph under the following conditions: injection volume of 20 µl, column temperature of 45°C, 0.01 N H_2SO_4 as the mobile phase used at a flow rate of 0.6 mL/min.

The microphotograph of the support surface was obtained using an optical microscope, model N107/T (Coleman, Santo André, SP, Brazil).

3. Results and discussion

The figure 1 demonstrates that is possible and promissing the use of Solid Fermentation for xylitol producion. The behavior of the curves of xylose comsumption and xylitol production is similar to when using the Submerged Fermentation method.

The cell growth behavior ilustrated in figure 2 was also similiar to when using the Submerged methods. During inicial times there is low cell growth but at the end of the fermentation the cell growth is highest.

The pH behavior observed (figure 3) was indeed expected by the fact that cells were metabolically active during the bioprocess and then consumed $(NH_4)_2SO_4$ as the nitrogen source and xylose as the carbon source. The former activity is fact responsible for continuously release of H^+ ions and the latter for CO_2 development.

Maybe the results about xylitol production can increase using a tampon solution that will permit a SF on a almost constant pH. The pH decrease in this kind of fermentation was more quick than when using the commum fermentation, probably because of the oxygen limited conditions that increase the consumption of nitrogen and carbon sources.

In futures experiments will be tried use artificial oxygen supply for increase the xylitol production.

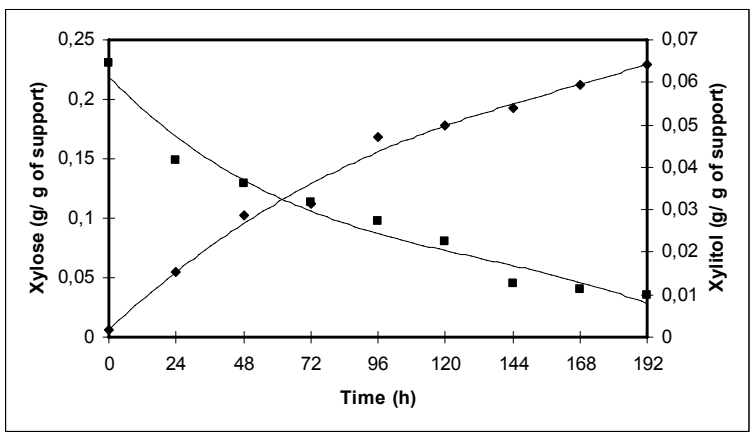

Fig 1 Time variation of xylose consumption and xylitol production during the Fermentation

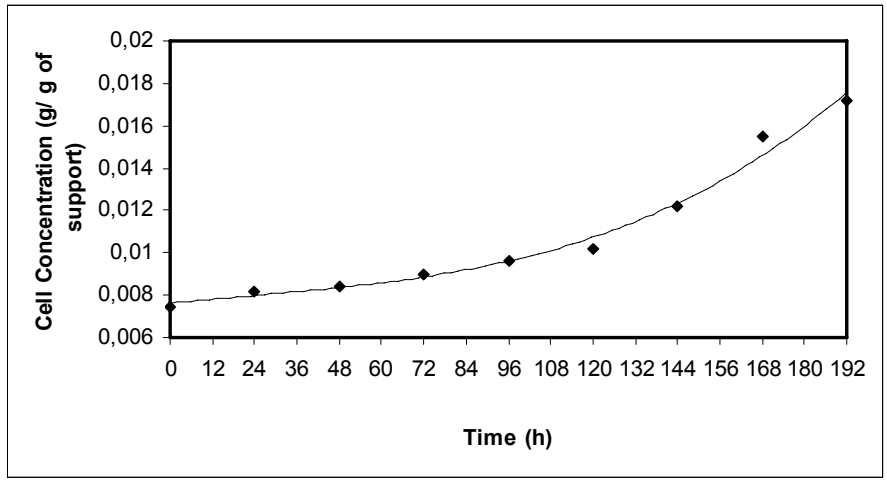

Fig 2 Time variation of Cell growth during the Fermentation

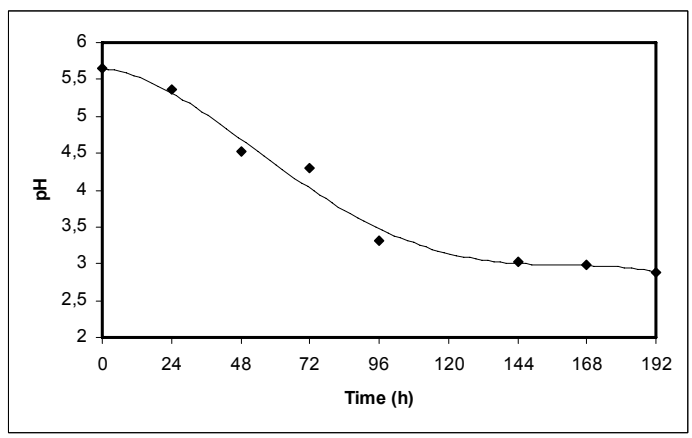

Fig 3 Time variation of pH during the Solid Fermentation for xylitol production

To confirm the occurrence of cell growth in this Solid Fermentation as well as to demonstrate the potential of the sugarcane bagasse as inert support, optical microphotographs were done at the end of experiments (192 h), which are illustrated in figure 4. It is noteworthy that cell growth was effective, as demonstrated by the uniform growth onto the whole surface of bagasse fibers.

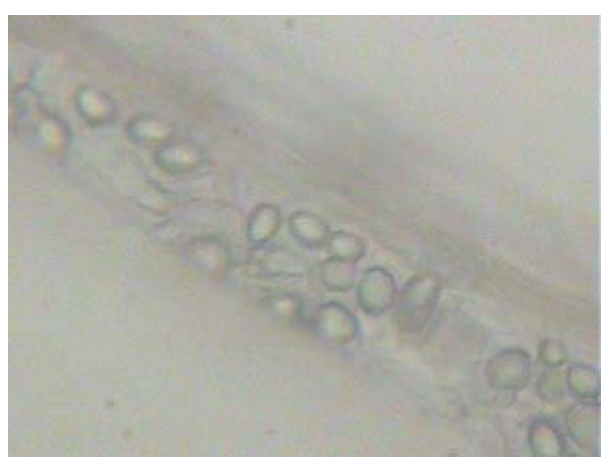

Fig 4 Optical microphotograph of Candida guilliermondii cells (400X) on sugarcane bagasse done after 192 h of fermentation.

4. Conclusions

Spite of the results about xylitol production comparing with others works using Submerged Fermentation appear not so good, this article demonstrated that is possible use Solid Fermentation as a new and alternative way for xylitol bioproduction.

Acknowledgements The authors gratefully acknowledge the financial support of FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

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Xylanase and cellulose free xylanase preparations from microscopic fungi isolated in the South Caucasus

E. Kvesitadze*, L. Kutateladze*, M. Jobava, N. Zakariashvili, I. Khokhashvili

Durmisnidze Institute of Biocemistry and Biotechnology, David Agmasheneblis Kheivani, 10km. Tbilisi 0159, Georgia

*Corresponding authors: kvesitadze@hotmail.com; kutateladze_20@hotmail.com; Telephone: (+99532) 528 026; Fax: (+99532) 528 026

The collection of microscopic fungi isolated from different ecological niches of the South Caucasus and accounting of more than 2000 cultures has been created. As a result of the selection, some strains of fungi including several extremophiles, actively producing xylanase under deep (submerge) conditions of cultivation have been selected. In the collection among xylanase producers dominate the representatives of the following genera: *Aspergillus, Penicillium* and *Trichoderma*. Among active producers of xylanase should be noted *Penicillium canescens* 41 (mesophile), *Aspergillus niger* A 7-5 (extreme halophile, thermotolerant), *Trichoderma viride* X1-6 (alkaliphile). These strains are distinguished by the following feature by having always accompanying xylanase, cellulase activities at zero level. Such strains are rare exception in nature. As a result of myco-toxicological studies it was stated that the selected strains are not toxic not pathogenic.

The physiology and some biochemical characteristics of the selected strains have been investigated, the nutrient media for each particular strain was optimized and conditions of growth were established.

The strain *Penicillium canescens* 41 reveals the highest xylanase activity at 27^0 C and pH 4,0; the strain *Aspergillus niger* A 7-5 at 40^o C, pH 6,0; *Trichoderma viride* X1-6 at 30^0 C, pH 7,5. As a result of optimization of the nutrient media the activities of xylanase are increased by 100, 75 and 60 %, respectively.

The preparation of xylanase has been obtained and the temperature and pH optimum for xylanase action has been revealed. For the xylanase produced by the strain *Penicillium canescens* 41 the optimal conditions for enzyme action are at temperature 45-50^0C and pH 4,4; for *Aspergillus niger* A 7-5 at temperature 65^0 C and pH 6,5 and that of *Trichoderma viride* X1-6, 50-55^0 C at pH 7,8-8,5. Above shown xylanases are significantly differing in their stabilities against increased temperature, acid and alkali conditions. These data indicate in difference of microscopic fungi xylanases stability against different extreme conditions and possibly in different areas of their application.

Keywords xylanase; extreme halophile; thermotolerant; alkaliphile; microscopic fungi.

Introduction

Microorganisms are considered to be the most prospective source for enzymes in both small and large scale production [1,2]. In addition to differing stabilities, especially in case of extremophilic microorganisms, enzymes from microorganisms have a number of advantages: unlimited wide spectrum of enzymes variety; short period of the time needed for the production of enzymes; possibility to increase the level of any particular enzyme biosynthesis via the selection of nutrient media and cultivation conditions; wide possibilities of gene cloning.

In the course of recent years, the interest towards the stable to different conditions enzymes and organisms of their producers has exstremely increased due to the wast potential of their application in enzyme production. Xylanase – hydrolizing hemicellulose and actively participaiting in wooden material degradetion atracts attention due to its application in a number of biotechnologies. Especially interesting are stable forms of this enzyme having increased resistance to different extreme conditions.

Materials and methods

The selection of microscopic fungi was carried out from strains isolated from different ecological niches of the south Caucasus. The action of temperature, pH and salt concentration (NaCl), on growth, development and production of xylanase by *Penicillium canescens* 41, *Aspergillus niger* A 7-5, and *Trichoderma viride* X1-6 has been investigated.

The microscopic fungi were cultivated in the temperature range of 5-55^0C, with the temperature interval of 5^0C, and at pH in the range from 2.0 to 10.0 with the 0.5 interval of pH of nutrient medium. The optimal values of temperature and pH provided the maximal fungi colony growth, is determined according to the colonies diameters and growth rate.

For the revelation of galophylicity of strains NaCI was added to nutrient medium in concentrations from 0.5M to 4.0M.

The active enzymes producers screening was conducted by submerged cultivation of strains. 10-Days conidia culture suspensions served as sowing material. The submerged cultivation of microscopic fungi strains was performed in 750 ml conical Erlenmeyer flasks on the thermostatic shaker at 180-200 rpm, at different temperatures, during 72 hours.

For the production of xylanases submrtged cultivation was carried out in a liquid medium of following composition, %: soy bean flour – 3.0; $NaHPO_4$ – 1.5; $(NH_4)_2SO_4$ – 0.2; KCl – 0.05; $MgSO_4$ – 0.015. In case of cellulase production, cultivation was carried out in a liquid medium, in %: microcrystalline cellulose-1.0, $NaNO_3$-0.3, KH_2PO_4-0.2; $MgSO_4 \times 7H_2O$-0.05; maize extract-1.5.

The activities of the enzymes were determined in the cultural filtrates of the strains.

For the detection of xylanase activity the Michael method was used [3].

Total cellulase activity was determined by the method of Ghose, based on the potential of cellulase to perform hydrolysis of insoluble substrates (filter paper) to redusible mono- and oligosaccharides [4]. Reducing sugars were estimated according the Somogyi-Nelson method [5,6].

Zoopathogenicity of the studied cultures was investigated by intravenous injection of the fungal suspension to rabbits [7]. The method of Berestetsky [8] was used to determine phytopathogenicity.

Toxicity was studied by Diekman technique [9].

The xylanase crude preparations have been obtained via the common methodology. Culture liquid was filtered, than cooled up to at 4^0C, and added acetone, or ethyl alcohol, or isopropanol in various quantities.

The temperature optimum of the action of xylanase was measured at temperature range from 20 to 80^0C.
pH optimum of the action of xylanase in a range between 2.0-10.0 has been determined.

Results and discussion

Since the natural conditions play a significant role in growth, development and physiological functions of microscopic fungi determining their variety [1, 10], the strains were isolated from different soil-climatic zones to obtain diverse experimental material. The collection of microscopic fungi isolated from different ecological niches of the South Caucasus and accounting of more than 2000 cultures has been created.

As a result of carried out studies among the various genera of microscopic fungi the excistance of active producers of xylanases and cellulases have been detected. As it has been determined some of them belong to group of fungi-extremophiles.

The strains active producers xylanase and cellulases were selected through screening of microscopic fungi cultures under submerged cultivation.

Among the cellulases and xylanases producers prevail the representatives of *Aspergillus*, *Trichoderma*, *Sporotrichum* and *Penicillium* genera. As it is seen in Fig. 1, among the producers of xylaneses and cellulases there are a number of thermophilic, alkaliphilic, acidophilic and halophilic microscopic fungi strains. As expected, most of xylanase producers simoltaneously synthesized cellulases. Especially high xylanase and cellulase activities were exposed by two thermophilic cultures: *Sporotrichum pulverulentum* 43 and *Chaetomium thermophile* I1.

It also should be underlined that the existence of several strains producers of cellulases free xylanases has been detected. Finally, for further experiments three physiologically differing strains were selected: *Penicillium canescens* B41 (mesophile), *Aspergillus Niger* A7-5 (extreme halophile/thermotolerant), and *Trichoderma viride* X1-6 (alkaliphile).

According to myco-toxicological studies it was stated that the selected strains are neither toxic nor pathogenic and maybe successfully used in a various industrial and agricultural processess.

The metabolic potential of fungi greatly depends upon the selection of the appropriate nutrient medium and the carbon source plays a special role in enzyme biosynthesis by the mechanisms of repression or induction in some enzymes synthesis [11, 12].

The temperature is extremely important factor for microorganism's growth and physiological activity, firstly the effect of temperature on the enzyme production was studied. Taking into consideration that the temperature optimum of growth of selected strains while their submerged cultivation was carried out within 30-55^0C, *Penicillium canescens* B41 revealed maximum of xylanase activity at 27^oC, *Aspergillus niger* A7-5 – at 40^oC, and *Trichoderma viride* X1-6 – at 30^oC.

The optimal pH for the growth of these strains has also been determined. Since the strain *Trichoderma viride* X1-6 is alkaliphile, the submerged cultivation of this strains was performed in ranges of pH from 6.5 to10.0. In spite of good growth of this strain in all range of pH the highest xylanase activity was determined at cultivation of the strain at pH 7.5, in more alkaline medium the xylanase activity was correspondingly reduced, while at pH 6.0 only 25% of the activity was retained.

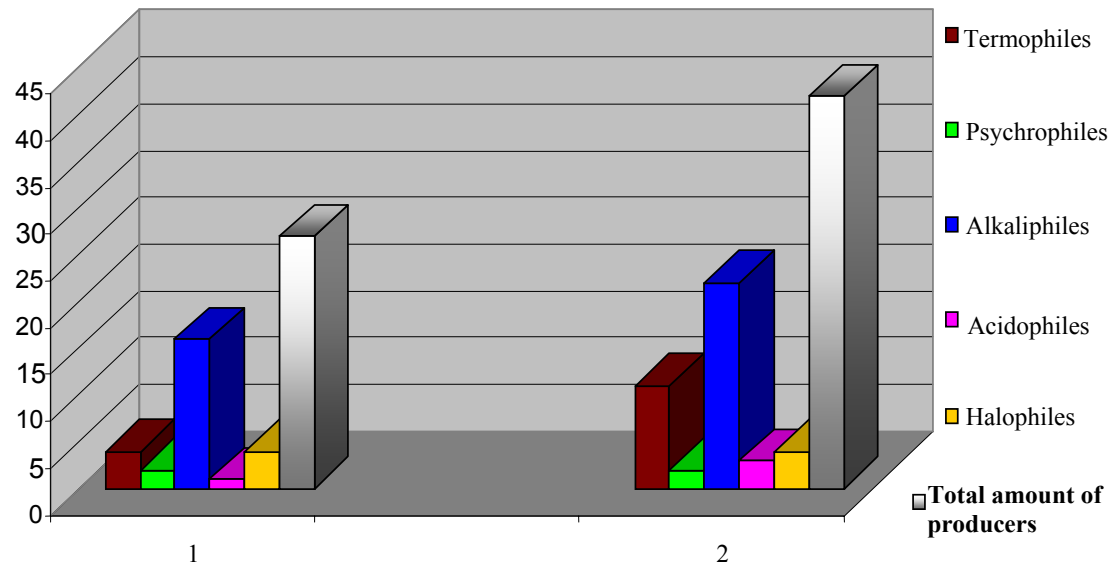


Fig.1 Extremophilic microscopic fungi producers of enzymes; 1.Producers of xylanases 2.Producers of cellulases

For strains *Penicillium canescens* B41 and *Aspergillus niger* A7-5 the optimal pH for growth, and xylanase production was correspondingly 4.0 and 6.0. Further optimization of growth conditions, and nutrient media compositaon, increased the activities of extracellular xylanase above 60%, are shown in Table 1.

Table 1 Microscopic fungi strains active producers of xylanases

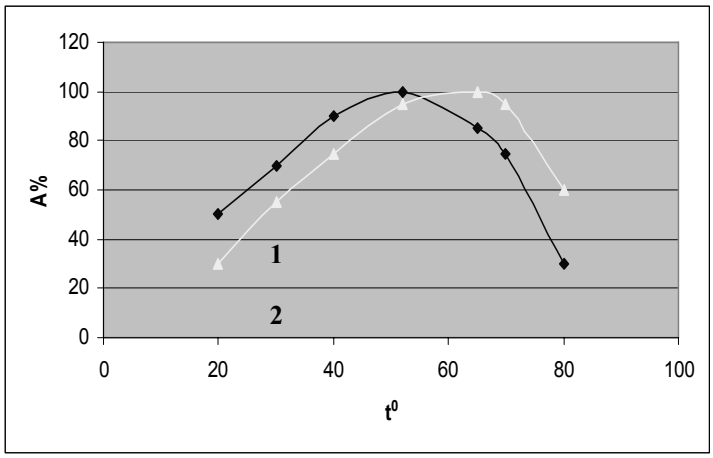
#	Strain	Inicial ativities, unit/ml	Activities unit/ml, after optimization of nutrient media	Activities unit/ml, after estimation in optimal conditions	% of increased activities
1	*Penicillium canescens* B 41	12.0	18.0	24.0	100
2	*Aspergillus niger* A 7-5	10.0	12.0	16.0	60
3	*Trichoderma viride* X 1-6	16.0	20.0	28.0	75

The comparison of physical-chemical characteristics of xylanases, produced by extremophes – *Aspergillus niger* A 7-5 and *Trichoderma viride* X 1-6 and mesophile – *Penicillium canescens* 41 exposed the differences in enzymes stabilities under different pH and temperature conditions.

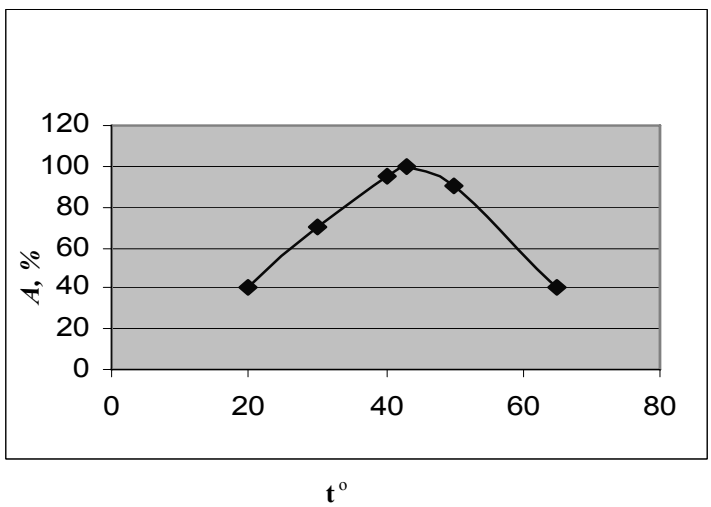
Ethyl alcohol was found as the best organic solvent for the isolation of xylanase crude preparations The temperature and pH optimums of xylanases of the selected strains were determined. The data are presented in Fig. 2a,b; and Fig.3 a,b. As it seen from the data of these figures the temperature and pH optimum of xylanase action from the strain *Penicillium canescens* B41, corresponds to 45-50^{o}C (incubation time 60min) and pH 4.4. For the xylanase from *Trichoderma viride* X1-6 the optimal temperature and pH was equal to 50^{o}-55^{o}C and pH 7.8-8.5. For xylanase of *Aspergillus niger* A7-5, optimal conditions of enzyme action are 65^{o}C and pH 6.5.

Stable cellulases and xylanases from the extremophilic fungi have the potential to convert agricultural wastes and plant indestructible substances into non-toxic, rich in protein biomass.

Xylanase of the extremophilic fungi are able to decompose and convert agricultural wastes containing xylan and cellulose. The lack of cellulase synthesis of the selected strains could be applied for high quality paper production.

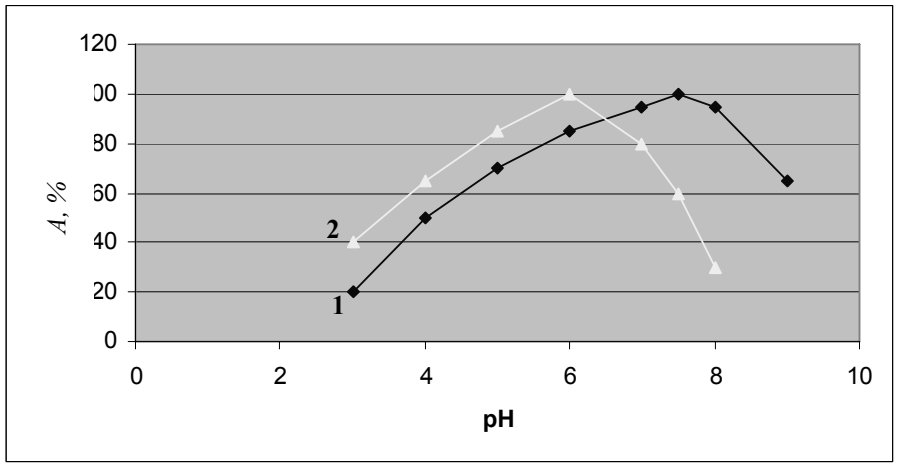


a)

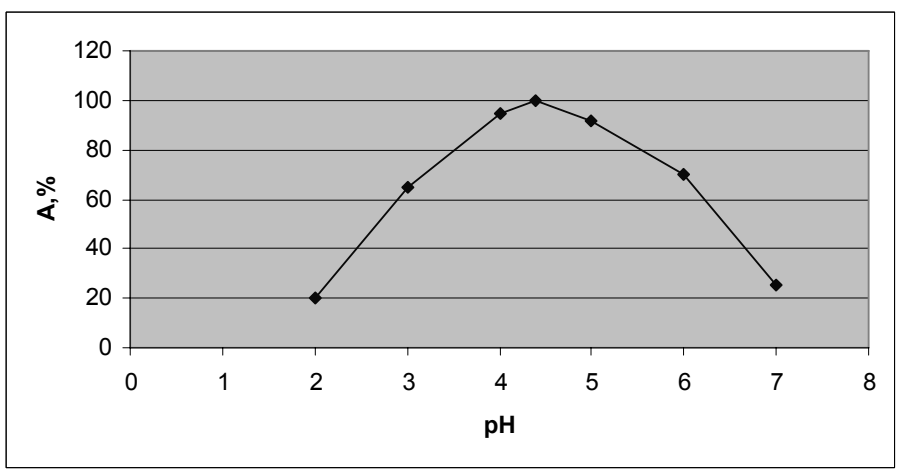


b)

Fig. 2 Effect of temperature on the action of xylanase a) 1.*Trichoderma viride* X1-6; 2. *Aspergillus niger* A 7-5 b) *Penicillium canescens* B-41



a)



b)

Fig. 3 Effect of pH on the action of xylanase a) 1. *Trichoderma viride* X1-6; 2. *Aspergillus niger* A 7-5 b) *Penicillium canescens* B-41

Acknowledgments This work was supported by a grant from the Georgian national Science Foundation.

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Xylose from *Eucalyptus globulus* wood as a raw material for bioethanol production

J.M.S. Rocha[1,*], L.F.F.Gomes[1], C.V.T. Mendes[2], C.M.S.G. Baptista[1], M.G.V.S. Carvalho[1]

[1] – CIEPQPF, Dep. Chemical Engineering, University of Coimbra, R. Sílvio Lima, Pólo II, 3030-790 Coimbra, Portugal

[2] – RAIZ – Forest and Paper Research Institute, Qtª. de São Francisco, Ap. 15, 3801-501, Eixo, Portugal

[*]Corresponding author: e-mail: jrocha@eq.uc.pt, Fax: +351239798703

Extracts from auto- and acid-hydrolysis of *Eucalyptus globulus* wood chips were used as fermentation media to produce ethanol by the yeast *Pichia stipitis*. pH adjustment with $Ca(OH)_2$ led to higher yeast activity than with NaOH, probably due to a detoxification effect. Furfural, in the range 0.1 to 0.8 g L^{-1}, did not act as an inhibitor of yeast growth. Substrate inhibition did not occur as well for xylose concentration as high as 60 g L^{-1}. Auto-hydrolysis extracts were submitted to a secondary hydrolysis. The use of different contents of auto- and acid-hydrolysis extracts as natural media, with the yeast previously adapted, showed that 75% (v/v) of acid-hydrolysis extract neutralized with $Ca(OH)_2$, led to 10 g L^{-1} ethanol concentration and a yield of 0.36 $g_{bioethanol}$ g_{sugars}^{-1}, under micro-aerobic conditions, at 30 ºC and pH 6.0. Although the maximum ethanol concentration reached was still very low, these results compare well to those obtained when using a synthetic medium with an equivalent concentration of xylose.

Keywords bioethanol, xylose, hemicellulose, *Pichia stipitis*

1. Introduction

Awareness of the limited fossil fuel reserves and the need to reduce carbon dioxide emissions produced by the combustion of these fuels, leads to an increasing interest in biofuels production. The use of bioethanol as a fuel, or as a fuel additive, could be a viable option to help fulfil the commitments of the Kyoto protocol [1]. At world scale, bioethanol is the most used biofuel due to its large production in Brazil and USA. Environmental impact due to intensive cultures and the increasing price of basic nourishment that is progressively being used for bioethanol production are showing that the sustainability of these processes is far from desirable. Therefore, research is now focused on other raw materials in order to reduce the environmental footprint of bioethanol/biofuels production [1, 2].

Cellulosic biomass is abundant, renewable, cheap and with a great potential to produce third generation bioethanol [3]. Apart from cellulose, hemicelluloses (around 25% w/w in wood) may have a potential for bioethanol production which, so far, has not been fully exploited [4, 5]. In the pulp industry, part of the hemicelluloses are dissolved during the wood cooking stage and burnt, together with lignin, in the recovery boiler to produce steam. Alternatives to this usage of hemicelluloses must be studied and this work aims to contribute to this goal. The hemicelluloses can be extracted before the cooking stage to undergo a fermentation process to produce value-added compounds, *e.g.* bioethanol. The extracts can be further hydrolysed to convert the hemicellulose polymeric chains into monosaccharides which can then be fermented. However, the liquid hydrolysates also contain water-soluble sugar degradation products such as furfural, hydroxymethyl furfural, and acetic acid. These compounds, along with lignin, can act cumulatively to inhibit the growth and fermentation activity of the microorganism selected [6, 7].

In Portugal, *Eucalyptus globulus* is the main wood species used to produce pulp and paper. The aim of this work is to use the hemicelluloses associated to this hardwood, which are mainly polymers of xylose, for bioethanol production. To extract them, an auto-hydrolysis and an acid-hydrolysis of wood chips were performed [8]. *Pichia stipitis* was selected due to its ability to ferment xylose into bioethanol [7].

2. Materials and Methods

2.1 Microorganism and culture media

Pichia stipitis DSM 3651, provided by DSMZ (Germany), was preserved on agar slants containing xylose (10 g L^{-1}), peptone (5 g L^{-1}), yeast extract (3 g L^{-1}) and malt extract (3 g L^{-1}), here called as synthetic medium.

Analytical grade xylose (Merck) and microbiology grade peptone (Fluka), malt extract (Sigma) and yeast extract (Fluka) were used.

To study the effect of potential inhibitors on yeast activity, different synthetic growth media were prepared by adding acetic acid (4 and 8 g L^{-1}), lignin (1 and 7 g L^{-1}) or furfural (0.1 and 0.8 g L^{-1}) to the medium described above. Reagents came from Riedel-de-Häen, Sigma-Aldrich and Merck, respectively.

Two kinds of liquid extracts from *Eucalyptus globulus* wood chips were used: i) one obtained from an auto-hydrolysis process, where temperature (150 ºC for 180 min) and the acids liberated from the wood (mainly acetic acid) provided the driving-force for the dissolution of hemicelluloses into the aqueous phase; ii) the other extract was obtained by a catalysed hydrolysis with sulphuric acid, 0.4% (w/w), at 140ºC for 180 min. Filtrated liquid extracts had xylose equivalent concentrations of *c.a.* 10 and 47 g L^{-1}, respectively. Different natural fermentation media included 100% (v/v) auto-hydrolysis extracts or 50, 75 and 100% (v/v) acid-hydrolysis extracts, neutralized with either NaOH or $Ca(OH)_2$. In order to increase the xylose content for the fermentation step, another extract from the wood auto-hydrolysis was submitted to a secondary hydrolysis [8], using 4 % (w/w) sulphuric acid for 3 h, and then also neutralized with NaOH. This hydrolysate had a xylose equivalent concentration around 16 g L^{-1}. The precipitates obtained after alkali addition were removed by centrifugation and vacuum filtration. All the hydrolysates were supplemented with peptone (5 g L^{-1}), yeast extract (3 g L^{-1}) and malt extract (3 g L^{-1}).

2.2 Fermentation conditions

Batch fermentations were carried out in 250 mL Erlenmeyer flasks, in an orbital shaker (Stuart S150) at 130 rpm and 30 ºC. 150 mL of sterilized medium and 10 mL of fresh inoculum were used. Media pH was adjusted to 5.5-6.5. Inocula were prepared using the standard media. Fed-batch fermentations were also carried out in a 5 L Biostat B Plus fermenter (Sartorius) at 30º C. pH was set at 6.5 by addition of NaOH or H_2SO_4, stirring speed was 150 rpm and the air flow rate varied between 0.05 and 0.5 L min^{-1}.

2.3 Analytical methods

Cultures were followed by cell density, xylose consumption and ethanol production. Total reducing sugars presented in different culture media, as equivalents of xylose, were determined by a colorimetric method using the dinitrosalicylic acid reagent (DNS) and a UV-VIS spectrophotometer (Beckman D.U. 650) at 540 nm. The culture media without xylose or hydrolysates were used as reference. The yeast growth was followed by measuring the optical density of the cells suspension (540 nm). The ethanol produced was analysed by HPLC (Knauer model K 301, RI detector), with a PL Hi-Plex Ca 8 μm column, at 85 ºC, using ultra-pure water as eluent at a flow rate of 0.6 mL min^{-1}. Samples were previously centrifuged and filtered through 0.2 μm pore size membrane filter (Whatman). The same chromatographic column was used to characterize the hydrolysates.

3. Results and Discussion

3.1 The potential use of xylose as a fermentable sugar

Xylose was firstly tested as raw material for bioethanol production. Batch and fed-batch fermentations were carried out with different xylose concentrations and air flow rates, in order to evaluate their influence on bioethanol production (Table 1). Besides xylose the media were supplemented with peptone (5 g L^{-1}), malt extract (3 g L^{-1}) and yeast extract (3 g L^{-1}). The theoretical yield is 0.52 $g_{bioethanol}$ g_{xylose}^{-1}. In the batch fermentation (A), the ethanol concentration reached 4.8 g L^{-1}, after 47 h. As the initial xylose concentration was 20 g L^{-1}, and was fully used, the corresponding yield and productivity were 0.24 $g_{bioethanol}$ g_{xylose}^{-1} and 0.102 $g_{bioethanol}$ $(L\ h)^{-1}$, respectively. Aiming to study the influence of air flow rate upon yield and productivity, this variable was changed in the fed batch fermentations. The outcomes of this strategy, however, were not the

Table 1 – Xylose fermentation conditions in Biostat fermenter (T=30 ºC, pH= 6.5, rpm=150 rpm).

Operation mode	Volume (L)	Initial xylose conc. (g L^{-1})	Xylose added (g)	Air flow rate (L min^{-1})
A - Batch	2	20	-	0.05
B - Fed-batch	2	40	40	< 0.05
C - Fed-batch	1.5	60	45	0.5[(1)]

[(1)]The air flow rate was reduced from 0.5 to 0.05 L min^{-1}, after 24 h of fermentation.

same in the two fermentations. Experiment B lasted for 163 h, the initial xylose concentration was 40 g L^{-1}, and 40 g was added at time 75 h. The maximum ethanol concentration was 19.4 g L^{-1}, with a yield of 0.35 $g_{bioethanol}$ g_{xylose}^{-1} and a productivity of 0.119 $g_{bioethanol}$ (L h)$^{-1}$. In fermentation (C) the initial xylose was 60 g L^{-1} and 45 g was added at time 120 h. The maximum ethanol concentration was 15.9 g L^{-1} with a yield of 0.31 $g_{bioethanol}$ g_{xylose}^{-1} and a productivity of 0.131 $g_{bioethanol}$ (L h)$^{-1}$. Being the initial air flow rate in experiment C tenfold higher than in B, and only reduced 24 h after the starting up, the results suggest that it is more efficient to provide microaerobic conditions from the beginning of the fermentation, favoring the yeast growth and the bioproduction of ethanol.

A second set of experiments carried out in Erlenmeyer flasks had previously revealed that the increase in xylose concentration from 10 to 60 g L^{-1} did not give rise to substrate inhibition.

3.2 Wood hemicelluloses extracts as substrates for the fermentation process

3.2.1 Influence of potential inhibitors on yeast growth kinetics

Apart from xylose the wood hydrolysis process also produces other chemical compounds, such as acetic acid and furfural that may inhibit the yeast growth. The ethanol production will be also affected. So, two synthetic media were prepared, one with additional acetic acid and the other with furfural. They were inoculated with *P. stipitis* in Erlenmeyer flasks and the yeast growth and xylose consumption were monitored to evaluate the toxic effect of those compounds. The results showed that the presence of furfural (0.1 and 0.8 g L^{-1}) did not affect the growth kinetics of *P. stipitis*, being the cell density increase and the xylose consumption rates very similar to those registered in a furfural free medium. The addition of furfural also had no remarkable influence on the media pH. However, the addition of acetic acid (4 and 8 g L^{-1}) lowered the pH to 4 and inhibited the yeast growth. pH was confirmed as an important factor to fermentation with *P. stipitis*. The presence of lignin, even at 1 g L^{-1}, was adverse to yeast growth rate, and must be separated, *e.g.* by precipitation, before the start-up of the fermentation process.

3.2.2 Acid-hydrolysates as substrate

The acid-hydrolysates pH was adjusted to 5.5-6.5 with NaOH or $Ca(OH)_2$ to check the influence of each alkali on the metabolic activity of *Pichia stipitis*. Fermentations were carried out in flasks with 50% (v/v) acid-hydrolysates (*i.e.* half-strength natural medium). The fermentation efficiency improved when $Ca(OH)_2$ was used instead of NaOH to correct the pH, likely due to a greater precipitation of inhibitory compounds, a process known as detoxification (Table 2). These were encouraging results when compared to those obtained with the synthetic medium, as they show a similar evolution of the cultures. The main differences were the longer fermentation time and the longer adaptation phase to the hydrolysate broth (~10 h), which can be overcome by a previous and gradual adaptation of the yeast to it.

The yeast was firstly adapted to hydrolysate (50% (v/v) treated with $Ca(OH)_2$) and used to inoculate culture media with 50%, 75% and 100% (v/v) of acid-hydrolysates. Figure 1a) shows that the yeast growth profile had no adaptation phase, with ethanol producing from the beginning. However, the ethanol obtained was very similar to that produced by the non-adapted strain. In the assay with 75% (v/v) acid-hydrolysate there was an adaptation phase that lasted *c.a.* 25 h, as shown in Figure 1b). Nevertheless the highest value of ethanol concentration was reached (10.3 g L^{-1} with a 0.36 $g_{bioethanol}$ $g_{xylose\ equivalents}^{-1}$ yield). However, with 100% (v/v) acid-hydrolysate (whole natural medium), ethanol production was not registered. Probably, the toxic compounds were not completely eliminated in the detoxification.

Table 2 Fermentation of xylose *vs* acid hydrolysates by *Pichia stipitis* (pH=6.5, 30ºC, rpm=150, in flasks).

Substrate	Alkali for pH adjust	xylose equivalents consumed ($g\ L^{-1}$)	maximum ethanol conc. ($g\ L^{-1}$)	yield ($g_{bioethanol}\ g_{xylose}^{-1}$)	productivity ($g_{bioethanol}\ (L\ h)^{-1}$)
D - Synthetic medium	NaOH	20.0	4.8	0.231	0.102
E - acid-hidrolysate 50%(v/v)	NaOH	19.7	3.9	0.196	0.081
F - acid-hidrolysate 50%(v/v)	$Ca(OH)_2$	21.2	6.5	0.377	0.087
G - acid-hidrolysate 50%(v/v)*	$Ca(OH)_2$	22.3	6.0	0.262	0.063
H - acid-hidrolysate 75%(v/v)*	$Ca(OH)_2$	33.7	10.3	0.358	0.071
I - acid-hidrolysate 100%(v/v)*	$Ca(OH)_2$	0	0	0	0

* - inoculum prepared with acid-hydrolysate 50% (v/v) treated with $Ca(OH)_2$.

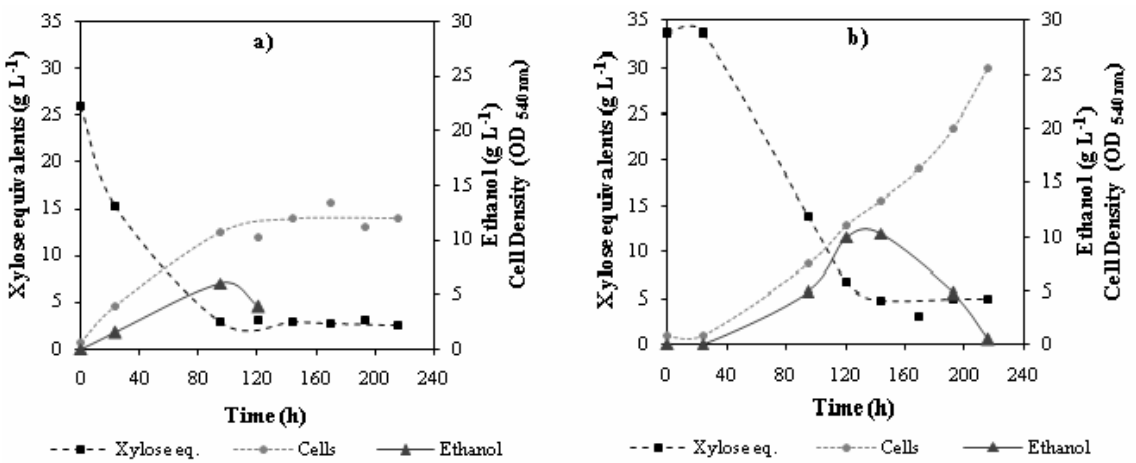

Figure 1 – Yeast growth profile, xylose equivalents consumption and ethanol production with a) 50% acid-hydrolysate medium and b) 75% acid-hydrolysate medium, both treated with $Ca(OH)_2$; experiments G and H in Table 2.

3.2.3 Auto-hydrolysates as substrate

The auto-hydrolysates pH was also adjusted with NaOH or $Ca(OH)_2$ before inoculation, when 100% (v/v) of this extract was used. The reducing sugars consumption and the ethanol production rates were very low and similar in both cases. So, the values of productivity and yield were also very small: 0.032 g $(L\ h)^{-1}$ and 0.27 $g_{bioethanol}\ g_{xylose\ equivalents}^{-1}$, respectively, for the auto-hydrolysate treated with NaOH, and 0.019 g $(L\ h)^{-1}$ and 0.26 $g_{bioethanol}\ g_{xylose\ equivalents}^{-1}$ for the one treated with $Ca(OH)_2$. Figure 2a) shows the fermentation profile of the auto-hydrolysate treated with NaOH. The very low ethanol production may be associated to the initial sugar concentration of auto-hydrolysates, which was very small compared to the acid-hydrolysates one. Besides, only half of the reducing sugars were consumed by the yeast.

The auto-hydrolysates were therefore submitted to a secondary hydrolysis process to convert part of the oligomers into fermentable monomers. Figure 2b) shows the cell density, the reducing sugars consumption and the ethanol production profiles. The initial sugar content was higher, as expected, but the maximum ethanol concentration was still very low (2 g L^{-1}) and started to decrease when no more sugars were consumed. The ethanol production rate was 0.017 g $(L\ h)^{-1}$ and the yield based on the reducing sugars consumed was 0.19 $g_{bioethanol}\ g_{xylose\ equivalents}^{-1}$. The ethanol concentration decreased towards the end of the fermentation, probably due to yeast metabolism and/or evaporation.

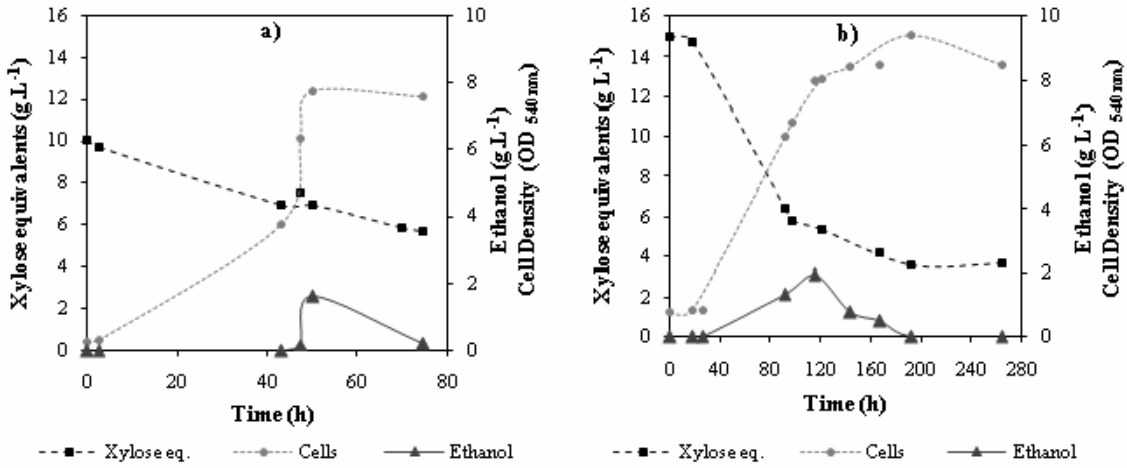

Figure 2 – Yeast growth profile, xylose equivalents consumption and ethanol production with auto-hydrolysates as culture media: a) directly obtained from wood extraction, b) after a secondary hydrolysis, both treated with NaOH.

4. Conclusions

Two kinds of fermentable liquid extracts, obtained from an auto- and an acid-hydrolysis of *Eucalyptus globulus* wood, were used to produce bioethanol by *Pichia stipitis*. The acid-hydrolysates are richer in xylose, leading to higher ethanol concentrations (10 gL^{-1}) and yields (0.36 g g^{-1}). A secondary hydrolysis was introduced to the auto-hydrolysis extracts to convert oligomers into monomers. Nevertheless, the maximum ethanol production was still very low. To reach the effectiveness of acid-hydrolysates, the auto-hydrolysates extracts must be added with xylose, or submitted to an alternative hydrolysis process. The work carried out with both extracts showed the need to select a pretreatment method to overcome the inhibitory effects over the *P. stipitis*, and to adjust and control the pH before and during the fermentation. The adjustment of pH with $Ca(OH)_2$ led to a better yeast performance than with NaOH, probably due to a greater detoxification. Furfural 0.8 g L^{-1} did not inhibit the fermentation and xylose concentration, as high as 60 g L^{-1}, did not induce substrate inhibition. The presence of lignin is adverse. The use of acid hydrolysates may be improved by the yeast adaptation process to the natural media.

Acknowledgments: The support of RAIZ, providing the wood extracts is gratefully acknowledged.

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β-lactam Resistance in *Escherichia coli* Isolates from Raptors in Spain

Pedro Jiménez-Gómez [1,*], José Esteban García de los Ríos [1], María José Pozuelo[1]; Francisco Llinares Pinel[1]; Rosa del Campo[2]

[1] Sección de Microbiología. Facultad de Farmacia. Universidad San Pablo C.E.U. Ctra. Boadilla del Monte Km, 5,300.
[2] Servicio de Microbiología. Hospital Universitario Ramón y Cajal, Ctra. Colmenar Km 9,1 Madrid 28034. SPAIN

[*]Corresponding author: Phone: 34-913368330; FAX-34-913368809; e-mail: pgimgom@ceu.es

A significant higher proportion of ampicillin resistance was detected in fecal *E. coli* isolates from 256 hospitalized raptors (42.5%) than in 643 wildlife ones (21%), mainly due in both cases to the presence of TEM-1. Hospital circulation of a particular multirresistant clone, that contain class I integrons, was also observed.

Keywords Wildlife, Captive, Raptors, Antibiotic Resistance, β-lactams.

Short communication

ß-lactamase production is the main mechanism of ß-lactam resistance in *Escherichia coli* isolates. A dramatic increase in the resistance rate to this antibiotics has been observed in the last decades, due to the presence of class A extended-spectrum ß-lactamases (ESBLs) (CTX-M or enzymes derived from TEM-1 and SHV-1) or plasmid mediated class C ß-lactamases (FOX and CMY, among others).

Several Spanish studies have been demonstrated the occurrence and dissemination of ESBLs among human clinical isolates from hospitalized patients as well as from those in the community (Valverde *et al.,* 2004). Prevalence of ß-lactamases in *E. coli* isolates from healthy (Briñas *et al.,* 2005, Riano *et al.*, 2006) or sick animals (Biñas *et al.,*2003) in Spain or Portugal has been also recently published although few studies of B-lactam resistance are focused on wildlife animals (Costa *et al.,* 2006), specially in raptors or migratory birds.

These wildlife animals are able to cross big distances in few time, and also their most important source of food are the corpses of farm animals. Both factor might be considered as a efficient mechanism of specific bacterial clones or resistant genes dissemination, and could explained the epidemiological differences for antibiotic resistance in animals in several countries.

GREFA is an Spanish organization that works for recovered the native fauna, and also has a Recuperation Hospital for sick birds, and parallel they have a reading breeding program of raptors. The aim of this work was to analyze the prevalence of β-lactam resistance and the molecular mechanisms implicated in fecal *E. coli* isolates from two different sets of birds: 643 wildlife birds, which arrive to hospitalization for first time, and 256 captive birds which were hospitalized or included in the rearing breeding.

One cloacal swab per raptor were collected at bird hospital admission, and before to the breed period (Table 1), and seeded in MacConkey agar (BioMerieux) plates supplemented with 32 μg/ml of ampicillin. To detected presence of multirresistant strains, cloacal swabs were also seeded in Chromogenic agar (BioMerieux) supplemented with 32 μg/ml of ampicillin, 4 μg/ml of ciprofloxacin, 64 μg/ml of doxycycline, and 128 μg/ml sulfamide. Plates were incubated during 48 h at 37ºC, and only one colony with *Enterobacteriaceae* morphology per bird was finally selected and identified using the APY ID 32 GN phenotypic proofs (BioMerieux, ATB Expression plus system).

Antibimicrobial susceptibilities for ampicillin, amoxicillin, amoxicillin/clavulanate, ampicillin/sulbactam, cefalotine, cefamandole, cefotaxime, and cefoxitin were determined by the agar dilution method (CLSI, 2005). For β-lactamase characterization, crude cell-free bacterial extracts by sonication were prepared from an Trypticase Soy broth (Difco, Detroit, MI) overnight culture. Isoelectric focusing was done using a Hoefer isobox and commercial LKB Ampholine PAG plates (pH range: 3.5-9.5 or 5.5-8.5). Gels were stained with the chromogenic Iode with G-penicillin. Controls strains carrying the SHV-l, TEM-1, TEM-2 and AmpC β-lactamases (pI=7.6, 5.4, 5.6, and 8.5, respectively) were used in each experiment.

Table 1. Raptor birds used in the study

Raptor Bird	Family	*Specie*	Wildlife	Captive
Golden Eagle	**Accipitidae**	*Aquila chrysaetos*	24	7
Bonelli's eagle		*Hieraetus fasciatus*	19	2
Short-toed eagle		*Circaetus gallicus*	32	9
Buzzard		*Buteo buteo*	48	8
Goshawk		*Accipiter gentilis*	14	5
Sparrowhawk		*Accipiter nisus*	36	12
Montagu's harrier		*Circus pygarus*	32	7
Red kite		*Milvus milvus*	50	12
Black kite		*Milvus migrans*	16	7
Black vulture		*Aegypius monachus*	27	16
Griffon vulture		*Gyps fulvus*	48	27
Kestrel	***Falconidae***	*Falco tinunculus*	32	17
Lesser kestrel		*Falco naumanni*	78	67
Barn owl	***Tytonidae***	*Tyto alba*	16	6
Eagle owl	***Strigidae***	*Bubo bubo*	32	8
Long-eared owl		*Asio otus*	24	7
Tawny owl		*Strix aluco*	31	6
Short-eared owl		*Asio flammeus*	24	8
Little owl		*Athene noctua*	23	6
Scops owl		*Otus scops*	37	19
		TOTAL	643	256

Pulsed-field gel electrophoresis (PFGE) was performed in the multirresistant strains as described previously (Gautom *et al*., 1977), digesting with *Eco*RI (Roche GmbH, Mannheim, Germany), and separated in a CHEF-DRIII system (Bio-Rad, La Jolla, Calif.), with the following conditions: 6 V/cm^2, 10-40 s, 27 h.

Genomic DNA from 24 selected antibiotics multirresistant strains were used as template for detection of Class I integrons (Levesque *et al.,* 1995), and positive amplicons were sequenced on ABI Prism 377 automated sequencer (Perkin-Elmer, Norwalk, Conn). The chi-square test were use for statistical analysis.

Serotyping identification of O antigens was carried out by a microtechnique described by Guinee *et al*, 1972 and modified by Blanco *et al*.,1998 with all available O antisera (O1 to O171). All antisera were adsorbed with the corresponding cross-reacting antigens to remove non-specific agglutinins.

A significant higher proportion of ampicillin resistant *E. coli* isolates were detected among in the captive raptors (42.5 %, 109 isolates from 256 birds), than in the wildlife raptors (21%, 135 isolates from 643 birds) ($p<0.0001$). Resistance to cefotaxime was not detected in any isolate. The isoelectric focusing results showed that pI band compatible with $bla_{\text{TEM-1}}$ was detected in 57% of the total ampicillin resistant isolates, whereas those compatible with AmpC were observed in 41% of the isolates. Susceptibility testing analysis revealed that AmpC hyperproduction was detected in less than 2% of isolates.

Considering the 899 cloacal swabs, a total of 24 multiresistant *E. coli* isolates (2.6%) were detected in the antibiotic supplemented plates; corresponding to 8 (1.2%) wildlife and 16 captive (6.2%) raptors ($p<0.0001$). A total of 12 different pulsotypes were detected among the 24 multirresistant isolates (Table 2).

Positive amplifications for the class I integron occurs in all 24 *E. coli* multirresistant isolates, observing different combination of antibiotic resistance genes: *dfr16-aadA2* (23 isolates, 51%), *dfr17-aadA5* (8 isolates, 18%), *dfrA1-aadA1* (9 isolates, 20%), and *sul3* (5 isolates, 11%) (Table 2).

As similar in humans, hospitalized raptors present more antibiotic resistance rates than wildlife ones. The PFGE data suggests a clonal circulation among the hospitalized birds, specially interesting in the case of pulsotype A that presents concomitant two different serotypes O8 and O119. Pulsotypes A, B and C are present in several species of raptors including captive and wildlife animals, suggesting low genetic diversity of *E. coli* isolates in those animals. The presence of several class I integrons indicates also that horizontal antibiotic resistant mechanisms transmission are possible.

Table 2. Characteristic of the 24 multirresistant *E. coli* isolates detected

Group	Raptor Bird	***Serotype***	***Pulsotype***	***Integron***
Captives	*Athene noctua*	O8,O119	A	*dfr*16-*aad*A2 + *dfr*17-*aad*A5
	Gyps fulvus	O8,O119	A	*dfr*16-*aad*A2 + *dfr*17-*aad*A5
	Gyps fulvus	O8,O119	A	*dfr*16-*aad*A2 + *dfr*17-*aad*A5
	Burhinus oedicnemus	O8,O119	A	*dfr*16-*aad*A2 + *dfr*17-*aad*A5
	Bubulcus ibis	ONT	B	*dfr*16-*aad*A2
	Falco naumanni	ONT	B	*dfr*16-*aad*A2
	Falco naumanni	ONT	B	*dfr*16-*aad*A2
	Athene noctua	ONT	B	*dfr*16-*aad*A2
	Otus scops	O16	G	*dfr*17-*aad*A5
	Buteo buteo	O18	I	*dfr*16-*aad*A2
	Falco naumanni	O20	J	*dfr*A1-*aad*A1
	Otus Scops	O81	L	*dfr*17-*aad*A5
	Ciconia ciconia	O114	F	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1
	Milvus migrans	O177	C	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1 + *sul*3
	Ciconia ciconia	O177	C	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1 + *sul*3
	Falco naumanni	O177	C	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1 + sul3
Wildlife	*Accipiter nisus*	ONT	B	*dfr*16-*aad*A2
	Hieraaetus fasciatus	ONT	B	*dfr*16-*aad*A2
	Gyps fulvus	ONT	B	*dfr*16-*aad*A2
	Gyps fulvus	O8	K	*dfr*A1-*aad*A1
	Hieraaetus fasciatus	O11	E	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1
	Gyps fulvus	O102	D	*dfr*17-*aad*A5 + *dfr*A1-*aad*A1
	Gyps fulvus	O173	H	*dfr*16-*aad*A2
	Gyps fulvus	O177	C	*dfr*16-*aad*A2 + *dfr*A1-*aad*A1 + sul3

Although presence of EBSL was never described in raptors, Briñas *et al*., recently reported it colonizing healthy and sick animals in Spain, which corresponding to human contact animals. Differences in antibiotic resistant epidemiology from different countries or regions might be explained by the special dispersion of colonizing isolates of migratory birds, and more studies concerning this issue should be monitored.

The presence the multirresistant strains in hospitalized birds could be associated to selection the therapeutic antimicrobial agents used, and also to the circulation of particular clones adapted to the hospital environmental. In the case of free-living birds, the multirresistant strains were mostly detected in carrioners, suggesting that this multirresistant strains could be disseminated by the food chain. Free-living carrioners diet is basically rest of farm animals, in which high rates of antimicrobial resistance are commonly described (Kang *et al*., 2005).

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Characterization and molecular epidemiology of *Enterobacter cloacae* clinical isolates producing extended-spectrum β-lactamases

Seok Hoon Jeong[1], **Il Kwon Bae**[1], **Seung Ghyu Sohn**[2], **Ha Il Jung**[2], **Young Jun An**[2], **Eui Suk Sohn**[2], **Jung Hun Lee**[2], and **Sang Hee Lee**[*,2]

[1] Department of Laboratory Medicine and Research Institute for Antimicrobial Resistance, Kosin University College of Medicine, Busan, 602-030, Republic of Korea
[2] Department of Biological Sciences, School of Biotechnology and Environmental Engineering, Myongji University, San 38-2 Namdong, Yongin, Gyeonggido, 449-728, Republic of Korea

[*]Corresponding author: e-mail: sangheelee@mju.ac.kr, Phone: +82 31 3306195

148 *Enterobacter cloacae* clinical isolates were identified from clinical specimens taken from adults hospitalized in Busan, Korea. Among 148 *E. cloacae* isolates, the resistance rates against ceftazidime, cefotaxime, and aztreonam were 50.0%, 29.6%, and 48.0%, respectively. A total of 50 isolates showed the resistance to more than one expanded-spectrum β-lactam agent. 82% (41 of 50) isolates showed positive results in the double-disc synergy test. Of these 41 isolates, one contained TEM-52 gene, 15 carried SHV-12 gene, three harboured CTX-M-9 gene, and 19 carried both SHV-12 and CTX-M-9 genes. The high prevalence (15.5%, 23 of 148) of CTX-M-9-producing *E. cloacae* isolates was first reported in Korea. Twenty-three *E. cloacae* isolates carrying CTX-M-9 gene showed 9 different profiles by enterobacterial repetitive intergenic consensus PCR, indicating that they were not originated from an epidemic clone.

Keywords CTX-M-9; SHV-12; TEM-52; Enterobacter cloacae; ESBL

1. Introduction

Enterobacter cloacae is a well-recognized nosocomial pathogen that causes significant infections [1]. This organism is intrinsically resistant to ampicillin and narrow-spectrum cephalosporins, and can acquire resistance to oxyimino-cephalosporins and aztreonam by mutational overproduction of the species-specific cephalosporinase or production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) [2, 3]. Among Enterobacteriaceae, *Escherichia coli* and *Klebsiella* spp. are well known to produce ESBLs but less is known about the incidence and phenotypes of ESBLs in *Enterobacter* spp. [3-5].

In recent years, studies were reported that significant proportion of *Enterobacter* spp. isolates showed multidrug resistance against third generation cephalosporins and aztreonam. The ESBL-production rate within *Enterobacter* spp. was ranged from 1% to 43% among hospitals in different countries [6-10]. Within ESBL-producing *Enterobacter* spp. isolated from Greece, 31% of *E. cloacae* produced CTX-M type ESBLs [6]. CTX-M-10 was also the most common type of ESBL among *Enterobacter* spp. in Spain [2]. An epidemic clone of TEM-24-producing *E. aerogenes* was recovered in France for 3 years follow-up study [11]. The ESBL production rate of *Enterobacter* spp. in Korea was 43% and SHV-12 was most commonly produced with only one isolate with CTX-M-14 like enzyme [10]. However, in Hong Kong, the overall ESBL production rate among *Enterobacter* spp. was 6.5% and differed by species ranged from 3.7% of *E. aerogenes* to 17.9% of *E. hormaechei* [12]. In Japan, only 1% of ESBL production was found in *E. cloacae* and CTX-M-2 was only type found [7].

In the present study, we attempted to determine the prevalence and epidemiology of ESBL-producing *E. cloacae* isolates from a university hospital in Korea. Because the scarceness of studies about ESBLs other than TEM and SHV type even high frequency of ESBL production among *Enterobacter* spp. in Korea, we also tried to identify CTX-M, PER, VEB, GES, and TLA type ESBLs within *E. cloacae* isolates in Korea.

2. Materials and methods

2.1 Bacterial strains and antimicrobial susceptibility tests

A total of 148 non-duplicated clinical isolates of *E. cloacae* were collected from patients admitted in Kosin University Gospel Hospital in Busan, Korea from January through September 2003. The isolates were identified

using conventional techniques and/or Vitek GNI card (bioMérieux Vitek Inc., Hazelwood, MO, USA). *E. coli* J53 Azide^R was used as the recipient strain for conjugation. *E. coli* ATCC 25922 was used as the MIC (minimal inhibitory concentration) reference strain. Antimicrobial susceptibilities to antibiotics were tested by the disc diffusion method on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) that were performed and interpreted according to the recommendations of the CLSI [13]. The impregnated antibiotics were amikacin, ampicillin, ampicillin-sulbactam, aztreonam, cefepime, cefotaxime, cefotetan, cefoxitin, ceftazidime, cephalothin, gentamicin, imipenem, and tobramycin (BBL, Becton Dickinson, Cockeysville, MD, USA).

2.2 Detection of ESBL production

The putative ESBL-producing isolates were examined by the double-disc synergy test (DDST) of Jarlier et al. [14]. The test was performed on Mueller-Hinton agar with discs of ceftazidime, cefotaxime, and aztreonam each containing 30 μg placed at distances of 20 mm (centre to centre) from a disc containing amoxicillin-clavulanic acid (20 μg/10 μg) in the centre of the plate. After overnight incubation at 37°C, ESBL production was determined by the presence of enhanced inhibition zones between each expanded-spectrum β-lactam (ceftazidime, cefotaxime, or aztreonam) and amoxicillin-clavulanic acid discs. MICs of the antimicrobial agents, including aztreonam, cefepime, cefotaxime, and ceftazidime were determined by the CLSI dilution method on Mueller-Hinton agar containing two-fold serially-diluted β-lactams for those which produce ESBLs [13]. An inoculum of 10^4 CFU per spot was delivered with a multipoint inoculator.

2.3 β-Lactam resistance transfer assays and analytical isoelectric focusing (IEF)

Mating experiments were performed as described previously with sodium azide-resistant *E. coli* J53 Azide^R as the recipient [14]. Cultures of each donor strain and *E. coli* J53 Azide^R grown in brain heart infusion (Difco Laboratories) were mixed and incubated for 18 h at 37°C. Transconjugants were then selected on MacConkey agar (Difco Laboratories) supplemented with ceftazidime (2 mg/L) and sodium azide (150 mg/L; Sigma, Louis, MO, USA).

Crude bacterial extracts were obtained from clinical isolates after centrifugation of sonicated culture as described previously [15]. Sonic extracts and sample buffer (TEFCO corporation, Tokyo, Japan) were mixed in same amount and run for electrophoresis on precast polyacrylamide gel (pH 3-10, TEFCO corporation) for 1 h at 100 V, 1 h at 200 V, and 40 min at 300 V. β-Lactamase activity was detected with 0.5 mM nitrocefin (Oxoid, Basingstoke, UK).

2.4 PCR amplification and DNA sequencing for ESBL genes

Searches for genes coding for the ESBLs including TEM, SHV, CTX-M, PER-1, VEB, GES, and TLA type enzymes were performed by PCR amplification. CTX-M enzymes were sub-classified into 5 groups, including CTX-M-1, -2, -8, -9 and -25 clusters, by amino acid sequence similarities [16]. In this study, we tried to detect genes encoding members of 4 groups except CTX-M-25 group. The oligonucleotide primer sets specific for the ESBLs used in the PCR amplifications were described previously [15]. The templates for PCR amplification in clinical isolates were a whole-cell lysate or a plasmid preparation. The PCR products were subjected to direct sequencing. Both strands of each PCR products were sequenced twice with an automatic sequencer (model 3730*xl*; Applied Biosystems, Weiterstadt, Germany), as described previously [15]. DNA sequence analysis was performed with DNASIS for Windows (Hitachi Software Engineering America Ltd., San Bruno, CA, USA). Database similarity searches for both the nucleotide sequences and deduced amino acid sequences were performed with BLAST at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.5 Enterobacterial repetitive consensus (ERIC) PCR

ERIC PCRs were performed in 50 μL volumes containing 10 ng of genomic DNA from clinical isolates, 4 mM $MgCl_2$, 50 pM of each primer; ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), 1.25 U of TaKaRa Ex *Taq* polymerase (TaKaRa, Otsa, Shiga, Japan), 0.2 mM of each dATP, dCTP, dGTP and dTTP in 25 mM TAPS [N-Tris(hydroxy)methyl-3-amino-propane sulphonic acid pH 9.3], 50mM KCl, and 1 mM 2-mercaptoethanol. Amplification was carried out using the following program: 95°C for 5 min followed by 35 cycles of 1 min at 47°C, 5 min at 70°C, and 1 min at 92°C. The final extension step was performed at 70°C for 10 min. The analysis of amplified products was performed in 2% Seakem LE agarose (BMA, Rockland, ME, USA).

3. Results

3.1 Antimicrobial susceptibility testing and ESBL production and transfer by conjugation

Among 148 isolates of *E. cloacae*, the resistance rate against ampicillin, ampicillin-sulbactam, cephalothin, cefoxitin, and cefotetan were 98.6%, 66.9%, 99.3%, 95.0%, and 59.8%, respectively. Expanded-spectrum β-lactams also had high resistance rates which were 50.0%, 29.6%, 4.4%, and 48.0% respectively to ceftazidime, cefotaxime, cefepime, and aztreonam. There was not a single isolate that showed resistance to imipenem. Amikacin, gentamicin, and tobramycin showed resistance rate at 5.4%, 17.5%, and 45.3%, respectively.

A total of 50 isolates that showed resistance to one or more of ceftazidime, cefotaxime, cefepime, and aztreonam was collected and investigated by DDST. Forty-one (82.0%) of 50 *E. cloacae* gave positive results and nine was negative in DDST (Table 1). In conjugation results, the ceftazidime-resistance of 29 isolates (58.0%) in 50 *E. cloacae* could be transferred.

3.2 Genotypes of ESBL and isoelectric focusing (IEF)

Among 50 isolates that had resistance to one or more expanded-spectrum β-lactams, there was not any isolates that showed positive results in PCR amplifications for the detection of CTX-M type genes other than CTX-M-9 group, PER-1, VEB, GES, or TLA type genes. TEM, SHV, and CTX-M-9 group genes were found in 45 (90%), 35 (70%), and 23 (46%) isolates in 50 *E. cloacae*, respectively (Table 1). The DNA sequencing results revealed that only one isolate had TEM-52 and the rest (44) were TEM-1, not an ESBL. All of 35 SHV and 23 CTX-M-9 group genes were SHV-12 and CTX-M-9 genes, respectively (Table 1). Nineteen (38%) of 50 resistant isolates had both SHV-12 and CTX-M-9 genes, 16 (32%) isolates had SHV-12 gene only, and four (8%) isolates had CTX-M-9 gene only. Ten (20%) *E. cloacae* of 50 resistant isolates did not have any of ESBL genes except that seven isolates had TEM-1 only. Five of 41 DDST positive isolates had no ESBL genes except that two isolates had TEM-1 only. ESBL genes were found in 4 of 9 DDST negative isolates: one isolate had SHV-12, one isolate had CTX-M-9, and two isolate had SHV-12 and CTX-M-9 (Table 1). TEM-1-, TEM-52-, SHV-12-, and CTX-M-9-harbouring isolates had corresponding IEF values at pI 5.3, 6.0, 8.2, and 8.0, respectively.

Table 1 Ambler class A ESBL genes detected from fifty *E. cloacae* isolates resistant to one or more expanded-spectrum β-lactams.

Double disk synergy test	Genotype of ESBL	No. of isolate
Positive (41)	$bla_{\text{TEM-52}}$ only	1
	$bla_{\text{SHV-12}}$ only	15
	$bla_{\text{CTX-M-9}}$ only	3
	$bla_{\text{SHV-12}}$ + $bla_{\text{CTX-M-9}}$	17
	Not detected	5
	Subtotal	41
Negative (9)	$bla_{\text{SHV-12}}$ only	1
	$bla_{\text{CTX-M-9}}$ only	1
	$bla_{\text{SHV-12}}$ + $bla_{\text{CTX-M-9}}$	2
	Not detected	5
	Subtotal	9
Total (50)	$bla_{\text{TEM-52}}$ only	1
	$bla_{\text{SHV-12}}$ only	16
	$bla_{\text{CTX-M-9}}$ only	4
	$bla_{\text{SHV-12}}$ + $bla_{\text{CTX-M-9}}$	19
	Not detected	10
	Total	50

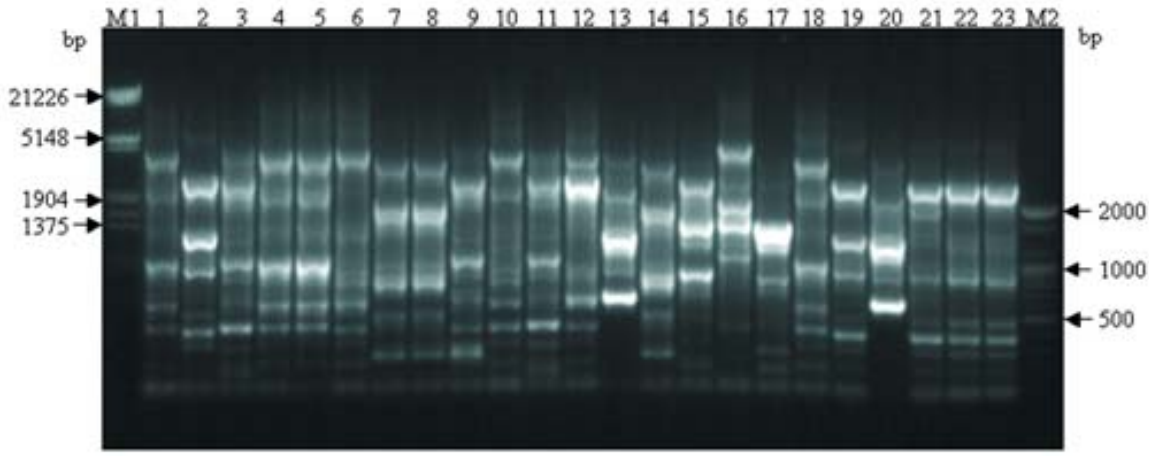

Fig. 1 ERIC patterns with primers of ERIC-1R and ERIC-2 with clinical *E. cloacae* isolates. Lane M1, *Hind* III/*Eco*R1-digested phage λ; lane M2, 100-bp DNA ladder; lane 1-23, CTX-M-9 ESBL-producing clinical *E. cloacae* isolates arranged in the order same as those of Table 2.

3.3 Characteristics of E. cloacae producing CTX-M-9

Twenty-three isolates produced CTX-M-9: nineteen did with SHV-12 and four did only CTX-M-9. The CTX-M-9-producing strains were isolated from 14 patients with malignant diseases including gastric, bile duct, esophageal, and pulmonary cancers, and 9 patients with benign diseases including intracranial hemorrhage, asthma, and tuberculoma (Table 2). The specimens were mostly from respiratory tract (n=13) and 6 were from body fluid, 2 from bile, and each one from urine and wound. These 23 CTX-M-9 positive isolates were classified into 9 groups by ERIC PCR (Fig. 1). Eight isolates were in Group A, 2 in B, 3 in C, each 2 in D and E, each one in F, G and H and 3 in I (Table 2). Group A strains were isolated in January (n=1), May (n=3), September (n=2), and November (n=1). Group I strains were isolated in December only from 1 patient in intensive care unit and 2 patients in general wards. MICs of ceftazidime, cefotaxime, and cefepime for 19 isolates of *E. cloacae* having both CTX-M-9 and SHV-12 genes were higher than that for isolates not having CTX-M-9 gene (Table 3).

Table 2 Characteristics of twenty-three E. cloacae isolates carrying CTX-M-9 gene.

Age/Sex	Ward	Date	Underlying Disease	Specimen	MIC (mg/L)				β-Lactamase gene type	ERIC Pattern
					ATM	CAZ	CTX	FEP		
28/F	GW	Jan	Stomach ca.	Ascitic fluid	128	64	128	8	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
48/M	GW	Feb	CBD ca.	Bile	128	64	64	8	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	B
33/M	GW	Mar	Asthma	Pleural fluid	256	256	128	16	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
67/M	ICU	May	ICH	CSF	64	32	64	4	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
62/M	ICU	May	Tuberculoma	Pleural fluid	64	64	128	8	bla_{SHV-12}+$bla_{CTX-M-9}$	A
76/M	GW	May	CBD ca.	Bile	64	32	32	2	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
50/F	GW	Jun	Tuberculoma	Respiratory	256	128	64	8	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	C
82/F	GW	Aug	Pneumonia	Respiratory	>256	256	128	32	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	C
74/M	ICU	Aug	Renal cell ca.	Respiratory	32	64	32	0.3	bla_{TEM-1}+$bla_{CTX-M-9}$	D
70/M	GW	Sep	Esophageal ca.	Respiratory	64	64	32	4	bla_{TEM-1}+$bla_{CTX-M-9}$	A
76/M	ICU	Sep	Esophageal ca.	Pleural fluid	>256	256	256	32	bla_{TEM-1}+$bla_{CTX-M-9}$	D
63/M	GW	Sep	Lung ca.	Respiratory	128	64	32	2	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
64/F	GW	Oct	HLD	Respiratory	128	128	64	8	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	E
69/F	OP	Oct	Pancreatic ca.	Ascitic fluid	16	128	64	0.5	bla_{SHV-12}+$bla_{CTX-M-9}$	C
56/F	GW	Oct	Lung ca.	Respiratory	>256	>256	128	32	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	F
64/M	ICU	Oct	Lung ca.	Respiratory	>256	>256	128	32	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	G
69/F	GW	Oct	Lung ca.	Respiratory	2	256	16	8	bla_{TEM-1}+$bla_{CTX-M-9}$	H
61/M	GW	Nov	ICH	Urine	8	32	256	4	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	A
48/M	GW	Nov	CBD ca.	Wound	>256	>256	64	16	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	B
58/M	GW	Nov	Esophageal ca.	Respiratory	128	128	16	2	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	E
46/M	ICU	Dec	Aneurysm	Respiratory	>256	>256	128	64	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	I
63/M	GW	Dec	Esophageal ca.	Respiratory	>256	256	32	16	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	I
62/F	GW	Dec	ICH	Respiratory	256	256	256	16	bla_{TEM-1}+bla_{SHV-12}+$bla_{CTX-M-9}$	I

Abbreviations: ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ERIC, enterobacterial repetitive intergenic concensus PCR; GW, general ward; ICU, intensive care unit; OP, outpatient; CBD, common bile duct; ICH, intracranial hemorrhage; HLD, herniated lumbar disc; CSF, cerebrospinal fluid.

Table 3 MICs of expanded-spectrum β-lactams in forty ESBL-producing *E. cloacae* isolates.

Allele (No. of isolates)	MIC (mg/L)											
	ATM			CAZ			CTX			FEP		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
$bla_{CTX-M-9}$ (4)	2->256			64-256			16-256			0.3-32		
bla_{SHV-12} (16)	4->256	128	>256	4-256	64	256	8-128	32	128	0.3-16	4	16
$bla_{CTX-M-9}$ +bla_{SHV-12} (19)	8->256	128	>256	32->256	128	>256	16-256	64	256	0.5-64	8	32
bla_{TEM-52} (1)	64			32			64			4		

Abbreviations: ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime.

4. Discussion

The resistance rate of *E. cloacae* to ceftazidime and aztreonam in this study was 50% and 48%, respectively and the rate was relatively higher than that (37%) in the study of Hong et al. which surveyed *E. cloacae* from 12 Korean hospitals in 2003 [17]. However, the resistance rate to cefotaxime and cefepime was 29.6% and 4.4% that were relatively lower than 34% and 12%, respectively, in the study of Hong et al. [17]. The DDST result showed that 27.7% (41/148) was positive, and the rate was similar as that (23.6%) in the study of Park et al. [18].

This study showed that TEM-52, SHV-12, and CTX-M-9 were the types of ESBLs found, but no CTX-M-1, -2, and -8 groups, PER-1, VEB, GES, and TLA types. TEM-52 is the frequently found ESBL from Enterobacteriaceae in Korea [19]. SHV-12 is also commonly produced ESBL type in *Enterobacter* spp. in Korea as true in this study that 35 isolates of *E. cloacae* produced SHV-12 (Table 1). CTX-M-9 was first reported in Spain from *E. coli* in 2000 and shared 88% amino acid identity with Toho-2 [20]. CTX-M-9-producing *E. coli*, *K. pneumoniae* and *E. cloacae* had been isolated in China [21], but in Korea, the high prevalence (15.5%, 23 of 148) of CTX-M-9-producing *E. cloacae* seems to be the first report. CTX-M-9 has more hydrolysis effect on cefotaxime than on ceftazidime. However, the isolates that produced CTX-M-9 in this study had the MIC of cefotaxime similar of ceftazidime (Table 2). These were probably due to the co-production with SHV-12 and/or chromosomal AmpC β-lactamase(s). The high prevalence of this CTX-M-9 ESBL suggests that diagnostic laboratories should screen for ESBLs with ceftazidime as well as cefotaxime; they should still perform clavulanate synergy tests on resistant isolates.

Among nine isolates that were negative in DDST, four isolates had ESBL genes: one with SHV-12 gene, one with CTX-M-9 gene, and two with both genes. The reason for negative ESBL detection seems to be the induction effect of clavulanic acid as an inhibitor of β-lactamase and the overproduction of chromosomal AmpC β-lactamase in *E. cloacae*, which resulted in defilade of the inhibition of clavulanic acid to ESBLs [22]. Five of 41 *E. cloacae* isolates that were positive in DDST did not have any of ESBL genes tested. Therefore, further development of ESBL detection methods would be needed.

ERIC PCR generates a characteristic genomic fingerprinting and used widely in the evaluations of outbreaks of *E. cloacae* as an alternative method of pulsed-field gel electrophoresis due to faster and easier procedures [23]. All 23 isolates that produced CTX-M-9 obtained distinct 4 to 8 bands ranging in size of 0.3 to 5.1 kbp by ERIC PCR and were classified into 9 groups. The most common type was Group A (8 of 23) and they were consistently isolated throughout the whole year. In December, Group I was identified in 3 isolates and suspected of a small outbreak in the hospital. Other groups were isolated throughout the year. However, further molecular epidemiological studies would be necessary to confirm the horizontal spread of integron- or insertion sequence-associated CTX-M-9 gene within the hospital.

In conclusion, the present study found out that the prevalence of ESBLs in *E. cloacae* seemed to be high in this hospital. Especially, the high prevalence of CTX-M-9-producing *E. cloacae* was first reported in Korea. SHV-12 and CTX-M-9 were the commonest types in *E. cloacae* and those with both enzymes were not rare. CTX-M-9-producing *E. cloacae* isolates showed diverse ERIC PCR profiles, indicating that they were not originated from an epidemic clone.

Acknowledgements This work was supported by research grants from the National Institute of Health (NIH) of KCDC in Republic of Korea, the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST), the Driving Force Project for the Next Generation of Gyeonggi Provincial Government in Republic of Korea, and

the Second-Phase of Brain Korea 21 Project. S.H. Jeong, I.K. Bae, S.G. Sohn, H.I. Jung, Y.J. An, and E.S. Sohn contributed equally to this work.

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Detection of *Porphyromonas gingivalis*, *Tanaerella forsythensis* and *Streptococcus intermedius* in dental plaque and saliva in young Children by Multiplex PCR

M A. De La Garza-Ramos[1], L. A. Galán-Wong[1], Caffesse RG[2], B. Pereyra-Alférez[1,*]

[1] Instituto de Biotecnología. Facultad de Ciencias Biológicas. Universidad Autónoma de Nuevo León. Pedro de Alba y Manuel L. Barragán S/N. Cd. Universitaria. San Nicolás de los Garza, NL. 66450. México.
[2] University of Texas Health Science Center at Houston, Houston, Texas, USA.

[*]Corresponding author: bpereyra@fcb.uanl.mx

Porphyromonas gingivalis, Tanaerella forsytensis and *Streptococcus intermedius* have been implicated as the main etiological agents of chronic periodontitis, but their role in young children is not clear. The goal of this study was to detect these bacteria in children population located at the Northeast of Mexico. A total of ninety six samples were analyzed and divided in three groups according age (years old): 1) Infants 0.5 to 4; 2) 4 to 8, and 3) 8 to 12. Samples were collected from saliva and dental plaques, resuspended in water and boiled for 10 min. Tubes were centrifuged and supernatant used directly as DNA template for multiplex PCR. Only sixteen were positive for some bacterium and they were distributed as follow: *P. gingivalis* and *T. forsythensis* were found in 5 samples; *S. intermedus* was found in 3 samples. The detection of the combination of *P. gingivalis* and *S. intermedius* was positive in 2 samples, while *T. forsythensis* and *S. intermeduis* in 1. In other 5 samples *T. forytensis* was detected. The detection of the combination of *P. gingivalis* and *S. intermedius* was positive in three samples and *T. forsythensis* and *S. intermedius*.

Keywords *Porphyromonas gingivalis*, *Tannerella forsythensis*, periodontal disease, young children, Multiplex PCR

1. Introduction

The majority of adults suffer from some degree of periodontitis, with severe disease affecting 5 to 20% of the population (Brown,*et. al.* 1996). Measurable attachment loss has been observed among 22% of 14- to 17-year-olds in the United States (Bhat,1991), suggesting that periodontal destruction begins very early. While over 300 species of bacteria have been identified in the oral cavity, *P. gingivalis* has been consistently associated with indicators of periodontal disease such as deeper pockets, alveolar bone loss, and attachment loss. Earlier studies which relied on cultivation for detection of *P. gingivalis* underestimated its prevalence in young subjects, seldom detecting it before puberty. More information on the acquisition and establishment of periodontal pathogens could help to elucidate the natural history of the development of chronic periodontitis and might provide information important for developing strategies for disease prevention. (Celeste W *et al., 1999).* Dental caries and periodontitis, as in many infectious diseases, require colonization by etiological pathogens before disease can occur. Improved microbiological methods suggest that colonization by pathogens associated with caries and periodontal disease occurs earlier than previously appreciated. Knowing the age at which these pathogens colonize the oral cavity will aid in our understanding of disease development, and in devising interceptive measures (Tanner *et. al.,* 2002) Alhough periontal disease is rare in healthy children, it is important to study the presence of some bacterial species that are potential periodontal pathogens in adults. Clinical studies have revealed the presence of *Porphyromonas gingivalis,* and *Streptococcus intermedius* in the periodontal disease. There are few data of the presence of these microorganisms in healthy. Their detection before puberty could help to identifying which children need to improved preventive progress in order to minimize the risk of periodontal disease. Periodontitis represents the most common gingival disease and it is relating to inflammation of the tissues supporting the teeth but is widely attributed to succession by polymicrobial communities. These microorganisms may attach to the tooth, to the epithelial surfaces of the gingival crevice or periodontal pocket (Mager *et al*., 2003). This chronic disease is characterized by leukocyte infiltration, release of tissue- destructive collagenase and the activation of the bone-destroying cells, osteoclasts (Kesavalu *et al*., 2007, Socransky *et al*., 1998). This affects the supporting tissues of the teeth, causing loss attachment of bone often resulting in tooth loss (Holt *et al*., 1999).

The purpose of this study was to identify and quantify the microorganisms from saliva and plaque in pediatric patients who assist to the clinic of pediatric Dentistry of the dental school at the Universidad Autonoma de Nuevo Leon.

2. Material and methods

Clinical Samples from patients. Clinical samples of sixteen healthy patients (10 males and 6 females; 4 infants, 4 prescholars and 9 nursing, fig.1) were evaluated. The sample was suspended in a 0.5µl tube containing 100µl of double distilled steril water and stored at -70ºC until use.
Unstimulated saliva was colected with a sterile cotton swab from the sublingual area of the mouth unil saturated. The DNA samples were obtained by placing the frozen tubes at 35ºC for 15 min, vortex and heated for 10 min at 94ºC. The cell lysate was centrifuged and a 35µl aliquot was used as DNA source for PCR assays. Alter prepare the suspension, they where added to a Standard PCR mix using DNA-primers.
The children wer separed in groups of age how is shown: Group 1. Infants. 6 months to 4 years old; Group 2 Prescholars 4-8 years old; Group 3 Young 8-12 years old
Oligonucleotide PCR primers design. Primers were designed by using all 16S rDNA sequences of *P. gingivalis* and *S. intermedius* available in the GenBank database. This survey was done using BLAST (National Center for Biotechnology Information) and the ClustalW program. Sequence of primers, their location within the respective gene sequences, and the expected sizes of their PCR products are shown in Table 1.
Clinical sample and PCR amplification. DNA samples were obtained by placing the frozen tubes at 37°C for 15 min, mixed in a vortex and heated for 10 min at 94°C. The cell lysate was centrifuged as mentioned before and a 35-µl aliquot was used as DNA source for PCR assays. PCR amplification was performed in a 50 µl volume containing 1X PCR buffer, 1.5 mM MgCl2, 2.5 U of *Taq* polymerase (Boehringer, Mannheim), 0.2 mM of each deoxynucleoside triphosphate (Boehringer, Mannheim), 0.2 µM of the primer and 35 µl of DNA sample. Amplification was carried out in a DNA thermal cycler(PerkinElmer model 2400). The process included 30 cycles of each of the following temperature programs: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. After that, an extra elongation step of 10 min at 72°C was added. A 10-µl aliquot of amplified samples from each PCR tube was placed in a electrophoresis gel containing 2% agarose or 4% polyacrylamide in 1 X TAE buffer (40 mM Tris- acetate, 1 mM EDTA (pH 8.0) for 1.5 h at 100 V, and stained with ethidium bromide.
Specificity and sensitivity of primers and PCR amplifications. The specificity and sensitivity of the primers and PCR conditions were evaluated by testing *P. gingivalis* ATCC 33277 and *S. intermedius* ATCC 27335, as well as the closely related *Tennerella forsythensis and P. intermedia* ATCC 25611 as positive controls; and the more distant *Escherichia coli*, *Proteus vulgaris, Bacillus thuringiensis* and *Bacillus subtilis* as negative bacteria. The oral strains *P. gingivalis* ATCC 33277, *S. intermedius* ATCC 27335, *P. intermedia* ATCC 25611 and *T. forsythensis*. The specificity of the primers was evaluated by testing positive and negative bacterial strains. DNA samples were obtained as reported elsewhere (Cerón *et al*., 1995). In brief, 103 cells from each strain were transferred to 100 µl of sterile double distilled water and placed for 10 min in a boiling water bath and then was used as a DNA sample in the PCR assays. Serial dilutions of the original culture in 1.0 ml of sterile double distilled water were made and the number of cells was determined by using a Petroff- Hausser bacteria-counting chamber; DNA samples were then obtained as described above.

3. Results

3.1 Detection of species by multiplex PCR and clinical data

A representative multiplex PCR result for clinical samples is shown in Figure 1, where several patients (lines 2-4) were positive for both microorganisms and one patient was positive for *S. intermedius* (line 5). The detection of *S. intermedius* and *T. forsythensis* or both was positive.

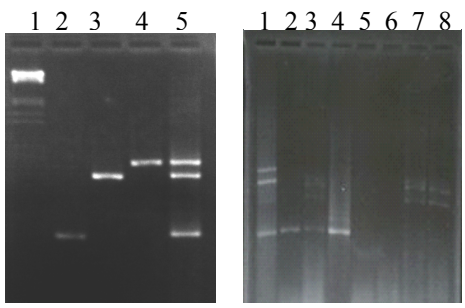

Figure 1: Representative PCR products with samples from pure cultures of strains collection. Panel A. Lanes: 1, DNA marker size; 2, *T. forsythensis*; 3, *P. gingivalis*; 4, *S. intermedius*; 5, Combination of 2-4. Panel B. Lanes: 1, Combination three bacterium; 2-8, clinical samples.

Table 1: Representative results for Multiplex PCR

Bacterias*	Percent (patients)
P.g	31.3% (5)
T.f	31.3% (5)
S.in	18.8% (3)

P. gingivalis and *T. forsythensis* were found in 5; *S. intermedus* was found in 3 samples. The detection of the combination of *P. gingivalis* and *S. intermedius* was positive in 2 samples and *T. forsythensis* and *S. intermeduis* was 6.25%, 1 sample (Table 1).

Table 2: Frequency by sex of detected bacterias

Name	**Female**	**Male**
P.g	2.6%	4.4%
T.f	3.3%	2.8%
S.i	2.3%	3.8%
P.int	1.0%	0%

**P.g*: *Porphyromonas gingivalis; T.f.*: *Tannerella foryithensis; S. i: Streptoccocus intermedius; P. int: Prevotella intermedia*

In order of gender the 62.5% the samples were found positive in male, 4.4% to *P.gingivalis*, 2.8% *T.forsythensis*, 3.8% *S .intermedius* and *S. intermedius* was negative. 37.5% was female, 2.6% to *P.gingivalis*, 3.3% *T. Forsythensis*, 2.3% *S.intermedius*, 1.0% was *P. intermedius* (Table 2) In order of age , *S. intermedius* was positive in 25% in lactants (unweaned baby), 19% Preschool children and 56% in young children (Table 3).

Table 3. Frequency by ages groups

Frecuency	Group	Percent (cases)
	Lactants	25% (4)
	preScholar	19% (3)
	Scholar	56% (9)
TOTAL		**100%**

Table 4: Frequency, mean and standard desviation of positives cases

	Frecuency	Mean	SD
female	62.5%	2.10	.88
Male	37.5%	2.67	.82

4. Discussion

The great diversity of biological surfaces in the oral cavity provides many ecological sites for colonization with a variety of oral bacterial species (Mager et *al*., 2003).

The main concern for DNA amplification through PCR techniques is related to the pretreatment of the sample prior to the PCR assay to get the DNA. It is well known that some biological samples may contain several PCR inhibitors and different methods have been suggested to eliminate or neutralize their activity (Amicosate *et al*., 1995, Mättö *et al*., 1998). In the present study, a cell lysate from a simple boiling step was used as the DNA source for multiplex PCR. Although Conrads *et al*. (1999) suggested that clinical samples should be processed with Chelex 100 resin prior to boiling, as a way to decrease potential PCR inhibition and increase sensitivity, our boiling step yielded positive and specific DNA amplification in 78% of the patients; this result was comparable with that found for *A. actinomycetemcomitans*, *T. forsythensis*, *P. intermedia* and *P. gingivalis* (Conrads *et al*., 1999; Tran & Rudney, 1999).Concerns with healthy volunteers, 23.5% of them were positive for *P. gingivalis*, but none for *S. intermedius*. This results agree with those reported by Griffen *et al*. (1998) and Leder *et al*. (2007), who found *P. gingivalis* in 25% and 24% of healthy subjects with chronic periodontitis. The great diversity of biological surfaces in the oral cavity provides many ecological sites for colonization with a variety of oral bacterial species (Mager et *al*., 2003). On the other hand in another study the frequency which bacteria were detected in randomly selected children from 6 to 36 months of age, the frequencies from tongues samples in children under 18 months were ; *S.mutans 70%, S. sobrinus 72%, P. gingivalis 23%, B. Forsythus*

(*T. forsythensis*) *11% and A. Actinomycetemcomitans 30%* with similar detection frequencies in children over 18 months. (Tanner *et al., 2002*) that was detected with checkerboard DNA Probe assay in the University of Washington, (Dental public Health Sciences). Although, we cannot assure the precise number of pathogen cells in plaque samples, our multiplex PCR assay detected as few as 8.2 cells of *P. gingivalis* and *S. intermedius* cells (data not shown) in pure cultures. This detection level is comparable to those obtained from other periodontal pathogens such as *A. actinomycetemcomitans* (Tran & Rudney, 1999, Jaffrey *et al*., 2003). In this sense, our approach is simple, highly sensitive, cost-effective and specific.

Another important feature of our work was to evaluate the presence of *S. intermedius* in oral samples from both healthy tissues and periodontitis lesions. Early reports recognized that this bacterium could be associated not only with periodontitis (Socransky & Haffajee, 1997; Kamma *et al.*, 1999; Nelson *et al*., 2003), but also with infections in deep sites such as liver and brain (Wiley *et al*., 1990).

Acknowledgement: This investigation was supported by: PROMEP/ 103.5/ 06/524 and PAICYT proyect-SA1194-05

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Herbal Antibacterial Liquid Soap Development against Bacterial Skin Diseases

S. Kongtun[*,1], and W. Suracherdkaiti[2]

[1] Department of Biotechnology,
[2] Department of Applied Statistics, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, 1518 Piboonsongkram Rd, Bangsue, Bangkok 10800 Thailand

[*]Corresponding author: e-mail: skongtun@yahoo.com , Phone: +662 913 2553

Antibacterial activities of herbal extract and essential oils in liquid soap were formulated and developed. Experiment was performed by selecting the herbs which were reported to have antibacterial activity. They are Kaffir Lime oil, Lemon Grass oil, King Orange oil, Mangosteen extract, Rinacanthus nasutus extract and Kaffir Lime juice extract. The essential oils and herbal extracts were obtained and tested by Minimum Inhibitory Concentration (MIC) for their activities against the bacterial causing skin diseases: *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginasa, Staphylococcus aureus* and *Staphylococcus epidermidis.* Results showed that Kaffir Lime oil, King Orange oil, Mangosteen extract and Kaffir Lime juice extract had significant effect against all bacteria. The combination between herbal extract and essential oil were then tested for their synergistic effect. Results revealed that the combination of Kaffir Lime oil-King Orange oil and Mangosteen extract-Kaffir Lime juice extract showed the synergism. Consequently, many types of herbal liquid soaps were formulated from the combination of herbal extract and essential oil. Natural and chemical surfactant liquid soap formulations were then retested MIC while those also were compared their activities toward the commercial antibacterial liquid soaps. Kaffir Lime oil, Mangosteen extract, Kaffir Lime oil-King Orange oil and Mangosteen extract-Kaffir Lime juice extract, were different. Combination of Kaffir Lime oil-King Orange oil and Mangosteen extract-Kaffir Lime juice extract showed higher efficiency than the single added of the Kaffir Lime oil, King Orange oil, Mangosteen extract and Kaffir Lime juice extract.

Keywords anti-bacterial agent, herbal extract, liquid soap

1. Introduction

Soap is a surfactant, which used with water for washing or cleaning. Soap can be obtained from the saponification reaction of oil, such as palm oil, olive oil, or coconut oil, and lye [1]. There are 2 types of soap, depend on a kind of lye use, which are soap bar or sodium soap and liquid soap or called potassium soap. Although liquid soap is usually more expensive than soap bar, but with the using of chemical surfactant to reduce cost and many advantages of liquid soap, such as easy to use, lower contamination, and several of formulation, all these reasons lead liquid soap products to widely use in everyday [2].

The development of liquid soap product is not all about detergent, the manufacturers usually added some substances for improve quality of products, such as moisturizing agents or antibacterial agents. Trichosan is a most popular for synthetic antibacterial agent added in soap products[3]. Although trichosan has been proven to be effective and the reported for non-toxic to humans and other mammals [4], but there is also evidence that trichosan may cause photoallergic contact dermatitis (PACD) [3], which occurs when the part of the skin exposed to trichosan is exposed to sunlight [5]. From this scientific report, some consumers refuse to use products containing trichosan and the manufacturers need to find some effective natural antibacterial agent.

There are some kinds of natural substances, including plant essential oil, and plant extract, which possesses an antibacterial activity. Some essential oils, such as tea tree oil, pine oil, and peppermint oil, are normally used as antibacterial agent in liquid soap and detergent products. In Thailand, there are some native plants which their essential oil or extract can be used as antibacterial agent in soap as well [6]. For this study, Kaffir Lime Oil (KLO), King Orange Oil (KOO), Kaffir Lime Juice (KLJ), Lemon Grass Oil (LGO), Rinacanthus nasutus extract (RNE) and Mangosteen extract (MTE) were added in liquid soap and tested for antibacterial effect of product.

2. Materials and Methods

2.1 Microorganisms and Plant materials

Microorganism: *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginasa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from Thailand Institute of Scientific and Technological Research. For plant materials, Kaffir Lime (*Citrus hystrix*) , Rinacanthus nasutus and Lemon Grass (*Cymbopogon citratus*) were collected from Nakorn Prathom province, Mangosteen (*Garcinia mangostana*) from Nakorn Sri-Thammarat province and King Orange (*Citrus reticulate*) from Chieng Mai province, Thailand.

2.2 Plant Extraction Methods

In this study, steam distillation was used for extract Kaffir Lime Oil (KLO), Lemon Grass Oil (LGO) and King Orange Oil (KOO). Rinacanthus nasutus fresh leave and fresh Mangosteen peel were extracted by juice extractor, and then concentrated by freeze dry technique until Rinacanthus nasutus extract (RNE) and Mangosteen extract (MTE) were obtained. Kaffir Lime fresh fruit was processed by hand press juicer, then Kaffir juice was centrifuged at 10,000 rpm for 30 min before use.

2.3 Herbal soap preparation

Two formulations of liquid soap were used in this study, which were natural formulation and chemical formulation. For natural liquid soap, palm oil, coconut oil and rice bran oil were mixed with aqueous solution of potassium hydroxide for saponification reaction. After reaction was completed, liquid soap was adjusted to pH 8 with citric acid and storage in glass container for 2 weeks. After that, it was mixed with herbal extract and continuously determined for anti-bacterial activity of herbal liquid soap. Ingredients of chemical liquid soap were sodium lauryl ether sulfate, triethanolamine lauryl sulfate, coconut fatty acid monoethanolamine, coconut fatty acid diethanolamine, 5-bromo-5-nitro-1.3-dioxane and water in this formula. One percent of herbal extracts were added in liquid soap and mixed spontaneously before antibacterial testing.

2.4 Antimicrobial assay

Anti-microbial activity of all crude herbal extracts and herbal liquid soaps were determined by agar diffusion method with Minimum Inhibitory Concentrations (MIC). The preliminary testing of antibacterial activities of all extracts against the bacterial causing skin diseases, which are *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginasa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were studied. Herbal extracts were combined and tested for synergistic antibacterial effects. The extracts or combination of extracts which showed efficiency were added in liquid soap as an antibacterial agent and then tested for antibacterial properties of finished products. The MIC of two commercial antibacterial liquid soaps were also determined. The comparison of antibacterial properties of herbal liquid soaps and commercial liquid soaps were studied.

3. Results and Discussion

3.1 Minimum Inhibitory Concentration (MIC) of Herbal Extracts and Combination of Herbal Extracts

From preliminary study of antibacterial activity of single herbal extract, the significant effects were obtained from Kaffir Lime Oil (KLO), Mangosteen extract (MTE) and King Orange Oil (KOO). There was no inhibitory effect from Lemon Grass (*Cymbopogon citratus*) and Rinacanthus nasutus (data not show). The combination between herbal extract and essential oil were then tested for their synergistic effect. The combination of Kaffir Lime Oil (KLO)-King Orange Oil (KOO) and Mangosteen extract (MTE)-Kaffir Lime Juice (KLJ) extract showed higher efficiency than the single addition of the Kaffir Lime Oil, King Orange Oil, Mangosteen extract and Kaffir Lime Juice extract. The antibacterial properties of essential oils were better than fresh herbal extract and the combination of herbal extract or essential oil were showed more effective effect than the single extract.

Table 1. MIC of herbal extracts and combination of herbal extracts

Bacterial species	Maximum (hr.)	PCA	Herbal extract	MIC
Bacillus subtilis	11	10^{-12}	KLO	1.56×10^{-2}
			MTE	6.25×10^{-2}
			KOO + KLO	3.13×10^{-2}
			MTE + KLJ	6.25×10^{-2}
Escherichia coli	12	10^{-12}	KLO	1.56×10^{-2}
			MTE	6.25×10^{-2}
			KOO + KLO	1.56×10^{-2}
			MTE + KLJ	6.25×10^{-2}
Pseudomonas aeruginasa	12	10^{-12}	KLO	7.81×10^{-3}
			MTE	1.56×10^{-2}
			KOO + KLO	1.56×10^{-2}
			MTE + KLJ	3.13×10^{-2}
Staphylococcus aureus	12	10^{-12}	KLO	7.81×10^{-3}
			MTE	3.13×10^{-2}
			KOO + KLO	1.56×10^{-2}
			MTE + KLJ	1.25×10^{-1}
Staphylococcus epidermidis	18	10^{-16}	KLO	1.56×10^{-2}
			MTE	3.13×10^{-2}
			KOO + KLO	3.13×10^{-2}
			MTE + KLJ	6.25×10^{-2}

3.2 Antibacterial effect of herbal liquid soap

The efficiency of antibacterial properties of 1% of single herbal extracts (Kaffir lime oil and mangosteen extract) in liquid soaps (both natural formula and chemical surfactant formula) were evaluated by inhibitory zone. From data in Table 2 and Table 3, the bigger in inhibitory zone size were obtained from chemical surfactant formula which mean the antibacterial properties of herbal extracts were more active in chemical surfactant liquid soap formula than natural formula.

Table 2. The efficiency of antibacterial properties of individual herbal extracts in liquid soaps

Bacterial species	Inhibitory zone size (mm)			
	Natural formula		Chem. surfactant formula	
	KLO	MTE	KLO	MTE
Bacillus subtilis	8.50	7.25	13.50	13.00
Escherichia coli	9.50	9.00	14.50	13.75
Pseudomonas aeruginosa	9.50	9.00	14.50	14.00
Staphylococcus aureus	10.50	9.50	15.00	13.50
Staphylococcus epidermidis	8.50	8.00	16.50	15.00

Table 3. The efficiency of antibacterial properties of herbal extracts combination in liquid soaps

Bacterial species	Inhibitory zone size (mm)			
	Natural formula		Chem. surfactant formula	
	KLO	MTE	KLO	MTE
Bacillus subtilis	10.15	11.05	19.25	23.75
Escherichia coli	9.25	11.00	22.00	23.50
Pseudomonas aeruginosa	10.25	10.00	22.50	21.00
Staphylococcus aureus	10.00	11.50	21.50	21.00
Staphylococcus epidermidis	9.50	10.00	21.00	21.50

3.3 The comparison of antibacterial properties of herbal liquid soaps and commercial liquid soaps

The efficiency of antibacterial properties between herbal liquid soaps and commercial liquid soaps were compared (Table 4 and Table 5). With the single herbal extract addition, the commercial products showed the higher inhibitory effect than herbal liquid soaps, but the combination of Kaffir lime oil and king orange oil in chemical liquid soap formula show the good synergistic effect in antibacterial properties and better than both commercial products.

The results from this study showed the synergistic effect of Kaffir lime oil and King orange oil and potential of the combination of these extracts in liquid soap. For development of antibacterial herbal liquid soap, chemical surfactant should be used to obtain the most effective products. Furthermore, the addition of Kaffir lime oil and King orange oil was not only for antibacterial agent, but they can be also used as aroma substances in the liquid soap products.

Table 4. The efficiency of antibacterial properties compared with individual herbal extract liquid soap and commercial liquid soap

Bacteria / Soap	Inhibitory zone size (mm)				
	B. subtilis	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*
Commercial [1]	10.00	11.00	10.50	11.75	9.75
Commercial [2]	13.75	17.00	17.75	16.25	17.25
Nat.formula+KLO	8.75	8.50	9.75	10.00	8.75
Nat.formula+MTE	8.25	7.75	9.50	9.00	8.00
Chem.formula+KLO	14.50	12.50	13.50	14.50	14.50
Chem.formula+MTE	13.00	13.50	13.00	14.00	12.50

Table 5. The efficiency of antibacterial properties compared with herbal extract combination liquid soap and commercial liquid soap

Bacteria / Soap	Inhibitory zone size (mm)				
	B. subtilis	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. epidermidis*
Commercial [1]	9.75	10.25	11.25	9.95	10.25
Commercial [2]	19.25	18.75	19.75	19.75	17.50
Nat.formula(KLO+KOO)	9.75	10.75	11.75	11.50	10.25
Nat.formula(MTE+KLJ)	9.25	9.75	10.75	10.00	9.75
Chem.formula(KLO+KOO)	21.25	23.75	20.75	22.25	22.50
Chem.formula(MTE+KLJ)	15.25	18.25	18.75	18.00	18.75

Acknowledgements Author would like to thank National Research Council of Thailand and Faculty of Applied Science, King Mongkut's University of Technology North Bangkok for budget and instruments which using for this study and Thailand Institute of Scientific and Technological Research for microbial substance support.

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Importance of High Levels Detention by *Enterococcus* Resistance (HLR) to Aminoglycosides in the Treatment of Serious Infections from Hospital

Marcelo M. Antunes[1,2,3,*]; Adriana A. Antunes[4,5]; Galba M. Campos – Takaki[5]

[1] Laboratório Central de Saúde Pública - LACEN, Recife/Brazil;
[2] Hospital Barão de Lucena, Recife/Brazil;
[3] Universidade Federal Rural de Pernambuco - UFRPE, Recife/Brazil;
[4] Pós-Graduação em Ciências Biológicas – UFPE, Recife/Brazil;
[5] Núcleo de Pesquisa em ciências Ambientais (NPCIAMB) - UNICAP, Recife/PE, Brazil; Rua Fernandes Vieira, S/N, Boa Vista, Recife/PE/Brazil.

[*]Corresponding author: mantunes@elogica.com.br/adri_antunes@yahoo.com.br

In the present study, a total of 40 enterococci samples, recovered from patients of a public hospital in the city of Recife, Pernambuco, Brazil, from March 2000 to September 2003 were analyzed. The primary goal of this study was to demonstrate the importance of the high level of resistance (HLR) to aminoglycosides from *Enterococcus* species. In order to achieve it, a sensitivity test was carried out by microdilution, using an automated technology from MicroScan, using gentamicin and streptomycin as standards. Among the 40 samples of *Enterococcus faecalis* tested, 16 were sensitive to streptomycin, gentamicin and no levels of HLR were detected for aminoglycosides in 40% of the samples; 10 were resistant to streptomycin, gentamicin and were detected HLR levels for all aminoglycosides in 25% of the samples; 8 were shown resistant to streptomycin and sensitive to gentamicin, with detected HLR levels for streptomycin in only 20% of the samples. Moreover, 6 samples were shown sensitive to streptomycin and resistant to gentamicin, with HLR detected levels for gentamicin and, consequently, also for tobramycin and netilmicin in 15% of the samples studied.

1. Introduction

Enterococcus spp. was first recognized as important ethiological agents of hospital-acquired infections in the middle of 1970s. Their rising pathogenicity was probably due to the use of third-generation cephalosporins, to wich these bacteria are naturally resistant [1]. They are Gram-positive, facultatively anaerobic cocci found in the gastrointestinal tract of most healthy humans and animals where they make up a significant portion of the normal microbiota [2,3]. They are released into the environment via the sewage where they can survive for long periods of time [2,3]. *Enterococcus*, in our days, represent the second leading cause of nosocomial urinary tract infections and the third leading cause of nosocomial bacteremia [4,5,6,7,8,9]. They include around 20 species, but most human enterococcal infections are caused by *E. faecalis* (80-90%) and *E. faecium* (10-15%) [10,11,12]. A few cases of human infections caused by other *enterococci* spp. such as *E. durans*, *E. gallinarum* and *E. casseliflavus* have also been reported [13,14,15,16,17]. There are several types and many different mechanisms for *Enterococcus* resistance. The most relevant are the inherent resistance, including the resistance to stable penicillins and low levels of aminoglycoside resistance, in addition to the acquired resistance, which includes the HLR – High Level of Resistance to aminoglycosides, characterized as a plasmid-mediated resistance caused by the expression of genes coding for enzymes that promote modifications in those antibiotics. The low level of the inherent or natural resistance to aminoglycosides by *Enterococcus*, difficults isolated treatments, although it allows their association to beta-lactam or to vancomicin, since those act at the cell wall level, promoting the aminoglycoside penetration. This activity, however, is not observed when *Enterococcus* shows a high level of aminoglicoside resistance (HLR) through the acquisition of enzymes responsible for the resistance, stopping presenting the classic synergic action between the antimicrobial and the aminoglycoside, leading to the probable therapeutic failure during the treatment of infections caused by *Enterococcus*. Isolates with high-level resistance to STRP (MIC > 2.000ug/ml) were first described in 1970, and, in 1979, strains highly resistant to GNT were reported [18,19]. Such strains are not killed by penicillin plus the respective aminoglycoside; therefore, to determine optimal therapy, clinically significant enterococci should be tested for high-level resistance to STRP and GNT. The abilities of commercial systems to detect high-level aminoglycoside resistance can vary. The synergy sensitivity screen (one well with 2.000ug of GNT per ml and one with 2.000ug of STRP per ml) is the conventional formulation.This activity is not observed when the *Enterococcus* presents one high level of resistance to the aminoglycoside (HLR - High Level Resistance) with MIC above of 2.000ug/ml through the acquisition of responsible enzymes for the resistance. This work's primary objective is to demonstrate the importance of the detection of the high level of *Enterococcus* resistance

(HLR) to the aminoglycoside with the purpose to evaluate the reliability of MicroScan rapid panels for detection of high-level resistance to aminoglycoside by enterococci. The synergies screen results provided by rapid and reformulated conventional MicroScan panels can offer a contribution to the treatment of severe infections caused by this microorganism.

2. Materials and methods

Microorganism

A total of 40 *Enterococcus faecalis* samples recovered from patients receiving medical care in a public hospital located in the city of Recife, Pernambuco state, Brazil, were collected from March 2000 to September 2003. The reference strains used as control were: *Enterococcus faecalis* ATCC 29212 (susceptible to vancomycin), *Enterococcus faecalis* SS 1332 (high level resistance to gentamicin and streptomycin, and vancomycin resistant) and *Enterococcus faecium* SS 1274.

Isolation and identification

For the isolation of *Enterococcus* faecalis a conventional method was applied by seeding blood culture flasks to enriched media (Blood Agar Base) and selective-indicators (Enterococosel Agar). Enterococci were identified using standard methods based on gram staining characteristics. For the physiologic characterization the following biochemical tests were used: catalase reaction, bile esculin, growth in 6.5 % NaCl, LAP and PYR, besides automated tests with panels pos combo for identification, including 20 biochemical tests.

Microscan panels

For HLR detection the sensitivity test was carried out by microdilution, using automated technology from MicroScan using gentamicin and streptomycin as standards, with MicroScan panels pos combo. Reformulated conventional (Pos Combo Type 6) and rapid (Pos Combo Type 1) panels were inoculated, incubated, and read according to the manufacturer's directions, except for the total incubation time for conventional panels that was 48 h. Both panel types were incubated in and interpreted with the MicroScan WalkAway-96 system (Dade Behring) (Figure 1). Rapid panels were read at 3.5, 4.5, 5.5, 7, 8, 11, and 15h; the results were reported when growth in the control wells was satisfactory. Conventional panels were read with the WalkAway system after 18 h and then examined visually. If one or both synergy screen wells showed no growth, the panel was re-incubated off-line at 35°C and read visually after incubation for a total period of 48 h. If a discrepancy existed between synergy screen results provided by the two panels or between results from a panel and the agar dilution, tests with both panels were repeated [20,21,22,23]. The minimal inhibitory concentration (MIC) was determined by microdilution method with Microscan panels pos combo, CLSI cutoff points were used to interpret MIC data. CLSI considered strains with a MIC ≤ 2μg/ml as susceptible, those with a MIC = 4μg/ml as intermediate, and those with a MIC ≥ 8μg/ml as resistant. Appropriate quality control was performed using *Enterococcus faecalis* and an investigational drug.

Figure 1: Following steps of Enterococcus isolation, identification and interpretation with MicroScan WalkAway-96 automated system (Dade Behring).

3. Results and discussion

Antibiotic resistance is one of the most serious global threats to the treatment of infectious diseases. In addition to resulting in significant increase in costs and toxicity of new drugs, antibiotic resistance is degrading our therapeutic arsenal from countries and hospitals, with the lack of control on antibiotic prescriptions increasing

the frequency of resistant organisms, which suggests a causal relationship. During past two decades, enterococci resistant to multiple antimicrobial agents have been recognized, including strains resistant to vancomycin, B-lactams and aminoglycoside, making it a formidable nosocomial pathogen. Such strains pose therapeutic dilemmas for clinicians. Thus it is crucial for laboratories to provide accurate antimicrobial resistance patterns for enterococci so that effective therapy and infection control measures can be initiated [24,25,26,27]. From a total of 40 *Enterococcus faecalis* samples tested, 16 were sensible to streptomycin, gentamicin and no detectable levels of HLR for aminoglycoside were observed; in 40% of the samples, 10 were resistant to streptomycin, gentamicin and HLR detected levels were observed for all aminoglycosides in 25% of the samples; 8 samples were resistant to streptomycin and sensitive to gentamicin, with HLR detectable levels only to streptomycin in 20% of samples, and 6 samples were sensitive to streptomycin and resistant to gentamicin, with detectable HLR levels to gentamicin and, consequently also to tobramycin and netilmicin in 15% of the samples studied, as shown in Table 1. According to our results, it is possible to conclude that the detection of *Enterococcus* strains showing high levels of resistance (HLR) is extremely important and must be included in the laboratorial routine, considering that these strains stop showing the classic synergic action between beta-lactams and aminoglycoside promoting failure of the treatment for severe infections, mainly hospital-acquired, caused by this microorganism.

Table 1 – Results from HLR (High Level Resistance) detection by *Enterococcus faecalis* using automated methodology from MicroScan WalkAway-96 system (Dade Berhing).

Enterococcus faecalis	**Gentamicin Synergism**	**Streptomycin Synergism**	**Percentual %**
16	S	S	40
10	R	R	25
08	R	S	20
06	S	R	15

4. Conclusions

Considering the results obtained in the present study, it is possible to conclude that the detection of *Enterococcus* samples with high level of resistance (HLR) is extremely important and should be included in the laboratorial routine, specially taking into account that 60% of the samples studied, showed, at least, one type of HLR, indicating that those samples stopped providing the classic synergistic action between β-lactams and aminoglycosides, promoting the failure in the treatment of severe infections, originated mainly from hospitals, caused by the afore mentioned microorganism. The presence of HLR for the two aminoglycosides markers was observed in 25% of the samples studied, what substantiate the concern in using the synergism of these these drugs.

Acknowledgements This work was carried out with financial support by Dade Behring, CAPES and CNPq.

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In vitro mechanism of xylitol action against *Staphylococcus aureus* ATCC 25923

Aline Siqueira Ferreira[1], **Nadia Rezende Barbosa**[2], **Durval Rodrigues Júnior**[1] and **Silvio Silvério da Silva**[1,3]

[1] Escola de Engenharia de Lorena, Universidade de São Paulo, CEP: 12610-810, Lorena-SP, Brazil
[2] Faculdade de Farmácia, Universidade Federal de Juiz de Fora, Juiz de Fora, MG – Brazil
[3] Faculdades Integradas Teresa D'Avila, CEP: 12.606-580, Lorena-SP, Brazil

The alarming increase of antibiotic resistant to bacterial pathogens makes it imperative to intensify the search for new means of combating such bacteria. Few works report carbohydrates action against pathogens. Xylitol, a sugar alcohol, can be appropriate for that combat, due to its effectiveness, safety and non-immunogenicity. The aim of this work was to investigate the antimicrobial and anti-adherent properties of chemically and biotechnologically obtained xylitol against *Staphylococcus aureus* ATCC 25923. The tested xylitol concentrations varied from 1.0% to 10.0% (w/v) for both assays. *S. aureus* was incubated in TSB medium containing xylitol at 37°C for 24h. A new and simple method was developed to evaluate its ability of inhibition of *S. aureus* adherence. On antimicrobial assays, xylitol did not show bactericidal or bacteriostatic activities against *S. aureus* and xylitol inhibited *S. aureus* adherence to a glass surface tester, both for at all tested concentrations. These results indicate that the anti-adherence property is associated with xylitol mechanism of action against *S. aureus*. Thus, xylitol reveleted as a new promising biotechnologically obtained drug to control diseases caused by *Staphylococcus aureus*. Through this mechanism, xylitol can explored to be applied as the chief component in a great variety of therapeutic formulations.

Keywords anti-adherence activity; antimicrobial activity; biotechnology; *Staphylococcus aureus* ATCC 25923; xylitol.

1. Introduction

Biotechnology drugs have become more popular over the last years. New products in advanced testing or under consideration for approval at the Food and Drug Administration include medications for osteoporosis, psoriasis, lupus, stroke, HIV (both treatments and vaccines), sickle cell disease, drug-resistant tuberculosis, hepatitis, chronic fatigue and rare genetic diseases. Over 370 biotechnology products are in late-stage development [1].

Most used common therapies are no longer adequate to treat infections since the resistance of microorganisms to antibiotics increases with its uptake. Therefore, it is imperative to search for different classes of substances that can combat pathogens growth and investigate how these new substances act against harmful microorganisms in order to improve the quality and specificity of researches which aim to discover drugs with potential applications and less undesired effects.

Carbohydrates are very useful due to safety, effectiveness and non-immunogenicity [2], which lessens microbial resistance. Xylitol, a five hydro-alcohol, is a monosaccharide successfully used as a sweetener [3], a therapeutic agent in glucose-6-phosphate dehydrogenase deficient people [4], at osteoporosis treatment [5], parenteral nutrition [6] and cystic fibrosis [7]. Besides, xylitol has reported action against *Streptococcus mutans* [8], *Streptococcus pneumoniae* [9], *Haemophilus influenzae* [10], *Clostridium difficile* [11], *Burkholderia cepacia* [12] and *Staphylococcus aureus* [13], and further applications. Moreover, there are few works about carbohydrates with anti-adherent properties against microorganisms [14–17] and considering they are not bactericidal, resistance to them is unlikely to develop. Few oligosaccharides can inhibit microorganism adherence, but no cases of monosaccharides with this property are currently found in literature. A new test to evaluate microorganism adherence to a surface (body test) was developed to verify if xylitol can inhibit this action. Unlike classical antibiotics that kill by interfering with some critical metabolic pathway specific to microorganisms, agents that block adhesion work by a non-selective mechanism [2]. So, they are the most indicated substances to control a wide variety of pathogens growth.

Xylitol is a substance that can be obtained both by chemical and biotechnological means, which is a relevant alternative that produces high-value compounds obtained by fermentation of lignocellulosic hydrolysates. The fermentation process occurs in a xylose-rich hydrolysate and traditional xylose-fermenting yeast is responsible to convert xylose into xylitol [18–22]. In this work, xylitol was produced by biotechnological means from fermentation of using wheat straw hemicellulosic hydrolysate by *Candida guilliermondii* yeast and purified according to [23].

The chief aim of this work is to elucidate xylitol mechanism of action against *Staphylococcus aureus* and provide useful information in order to designate more specific functions to this compound for future clinical applications.

2. Material and Methods

2.1 Microorganism

Staphylococcus aureus ATCC 25923 (American Type Culture Collection) was conserved in skim-milk and grown in Tryptic Soy Agar (TSA) (acumedia®, Canada) at 37°C for 24 hours.

2.2 Xylitol

Chemical xylitol (purity > 98 %) purchased from Fluka BioChemika (Switzerland) was used. Biotecnological xylitol (95 % of purity) was obtained as described in [23]. This compound was previously dried at 60 °C for 2 h before weighting.

2.3 Antimicrobial assays

A bacterial suspension with sterilized saline was prepared from the bacteria grown in TSA (acumedia®, Canada) and standardized aseptically so that the suspension had a 25 % transmittance in saline at λ = 580 nm (SHIMADZU®, UV-Vis, mini1240, Japan). Saline was used as blank. Seven 10-fold serial dilutions were made and 1.0 mL of each dilution was inoculated by pour-plate using Standard Count Agar (SCA) (acumedia®, Canada). Petri dishes were prepared in duplicate and incubated at 24 h at 37 °C.

Colonies were then counted and the suspension containing $10^3 - 10^4$ CFU/ mL (colonies forming unit/ mL) was re-prepared to use. Assays consisted in the addition of 1.0 mL of this suspension and 1.0 mL xylitol at 1.0% (w/ v), 5.0% (w/ v) and 10.0% (w/ v) in 4.0 mL of Tryptic Soy Broth (TSB) (Merck, Germany) twice-concentrated. Positive control media received only 1.0 mL of bacterial suspension and negative control media consisted of non-inoculated TSB. Tests were carried out in triplicate. Growth was allowed to happen for 24 hours at 37 °C, and was further evaluated by the turbidity of the broth (adapted from [24]).

2.4 Cellular adherence assays

This methodology was developed in accordance with [25]. *Staphylococcus aureus* ATCC 25923 was transferred to Baird-Parker (BP) (Difco™, United States) and grown at 37 °C for 24 h. Colonies were resuspended and 200 µL of the suspension were transferred to 5.0 mL of TSB and grown at 37 °C for 24 h.

Afterwards, an aliquot of 1.0 mL was transferred to a test tube containing 9.0 mL of Phosphate Buffer Saline (PBS) at pH 7.4. The Optical Density (OD) was measured in a spectrophotometer at 600 nm. PBS was used as blank. The volume of solution which had 0.01 OD was used as inoculum. This volume was added to a 50 mL Falcon tube containing 25.0 mL of TSB, a body test and 50 % (w/ v) xylitol to achieve the following final concentrations: 1.0 % (w/v), 5.0 % (w/v) and 10.0 % (w/ v). Fifty percent glucose was added to the Falcon tube containing the positive control medium to achieve 5.0 % (w/v) as final concentration. A 1:1 (v/ v) ether: chloroform solution was used to remove fats from the body test. Bacteria were grown at 37°C for 48 h.

Each body test was removed and placed into another sterilized 50 mL Falcon tube. Bacterial suspensions were centrifuged for 30 minutes, at 4000 g. Body tests were aseptically washed twice with sterilized PBS at pH 7.4 and then individually sonicated twice for 10 minutes at 40 KHz (UNIQUE, USC1400, Brazil) with 10.00 mL PBS. Afterwards, three 10-fold serial dilutions were prepared with each sonicated body test and 1.0 mL of each dilution was inoculated in Petri dishes with TSA by pour-plate, in duplicate, and incubated at 37°C, during 24 hours. After sonication, body tests underwent another twice-washing with 10 mL PBS and were fixed with glutaraldehyde (grade I) (Sigma, United States) 1.0 % for 12 hours. At the same time, *S. aureus* pellets obtained by centrifugation were washed twice with 2.0 mL PBS and resupended with 10.00 mL PBS. Each suspension (100 µL) was inoculated in SCA (acumedia®, Canada) by drop-plate in duplicate, and incubated at 37 °C for 12 hours. Afterwards, body tests were washed twice with PBS and immediately dehydrated by ethanol at increasing concentrations: 50%, 70%, 80%, 95% and 100% (v/ v), remaining for 20 minutes in each solution. Finally, these body tests were dried with care so that the center of the body test did not touch anything. Drying was performed at room temperature, and body tests were adequately conserved for Scanning Electron Microscopy (SEM).

2.4.1 Scanning Electron Microscopy

After completely dried, body tests were metallized by a very fine 10 nm gold layer with an ion sputter (MED 020, Baltec, USA) and examined by SEM (LEO1450VP, England), using high vacuum and retro- spared secondary electrons.

3. Results

3.1 Antimicrobial assays

No alteration was observed in the broth clearance of the negative control media. Positive control media was turbid, which indicates bacterial growth. In all three concentrations of the assay the broth was turbid, indicating that both chemical and biotechnological xylitol did not inhibit *S. aureus* growth (Fig. 1).

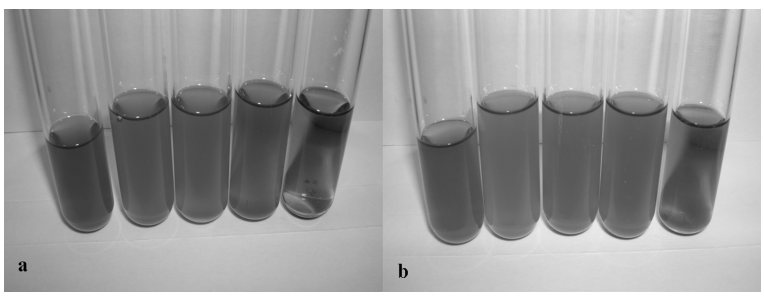

Fig. 1 Effect of chemically (a) and biotechnologically (b) obtained xylitol concentration on viability *S. aureus* cells. 1st tube: positive control, 2nd, 3rd and 4th tubes: assays tubes (1.0, 5.0 and 10.0 % xylitol), 5th tube: negative control.

3.2 Cellular adherence assays

It was observed that xylitol does not inhibit *S. aureus* ATCC 25923 growth, as shown by the uncountable CFU mL^{-1} found in all Petri dishes. This result is in accordance with those observed in the antimicrobial tests.
Fewer colonies were found in Petri dishes incubated with sonicated body tests cultivated with glucose (Table 1), which indicates that these microorganisms had fixed in the body test. In broths containing xylitol, more colonies are found at Petri dishes when compared to glucose and there were no cells adhered in body tests.

Table 1 Bacteria count (CFU. ml^{-1}) of each sonicated PBS 7.4 and its respective dilutions, inoculated in TSA (duplicate) on Petri dishes and incubated at 37°C for 12 hours.

Substance	Concentrations (% w/ v)	Dilutions			
		10^{-2}		10^{-3}	
Glucose (positive control)	5.0	29	17	4	2
Chemical xylitol	1.0	44	48	4	10
	5.0	120	132	29	18
	10.0	441	412	53	36
Biotechnological xylitol	1.0	108	98	12	8
	5.0	232	244	34	25
	10.0	862	943	180	185

Body test scanning electron microphotographies (Fig. 2 and 3) strengthened the anti-adherence mechanism of xylitol action. Many *S. aureus* cells were observed in positive control media, but they were not observed in the body tests treated with xylitol. Body tests were examined throughout its entire surface.

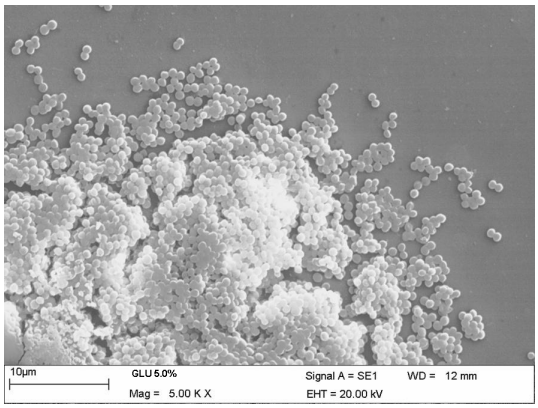

Fig. 2 Cells of *S. aureus* cultivated on TSB medium containing glucose (5.0 %) adhered to body test. Scale bar, 10µm.

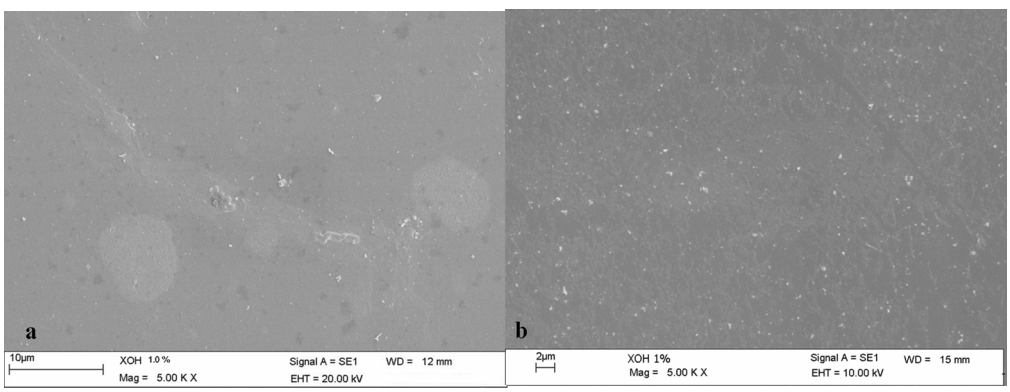

Fig. 3 Absence of cells of *S. aureus* cultivated on TSB medium containing chemically (a) and biotechnologically (b) obtained xylitol (1.0 %) adhered to body test. Scale bar, 10 µm.

This work evidenced that although xylitol does not inhibit *S. aureus* growth, it inhibits its adherence to a surface.

4. Discussion

Despite the reports that xylitol inhibits pathogenic microorganism growth [9, 26 – 29], it was observed that xylitol, in fact, inhibits the adherence of cells to a surface. Hence, xylitol can be extremely valuable for the control and treatment of infections considering that the majority of infectious diseases are initiated by adhesion of pathogenic organisms to cells and mucosal surfaces of the host [15]. Cellular adhesion is mediated by bacterial surface lectins and can be inhibited by sugars. A probable mechanism of action is binding to complementary carbohydrate constituents of glycoproteins or glycolipids on the surface of the host tissues, thus disabling pathogenic microorganisms to adhere cell surface.

This is the first time a work compares the action of chemical and biotechnological xylitol on microorganism growth. We hypothesized that the reason of the approximate half number of CFU/ mL counted when chemical xylitol was used compared to biotechnological xylitol is due to the 5 % impurities present in the assay, which can be acting as coadjuvant. An alternative explanation is the improvement on bacteria growth since these impurities are mainly carbohydrates. This hypothesis is less probable because of the low concentration of these impurities, especially because no differences were observed in microorganism growth during both essays.

Xylitol does not inhibit *Staphylococcus aureus* growth, but it inhibits *S. aureus* adherence to a surface. Elucidating xylitol mechanism of action is an important step to study more applications for this compound.

Acknowledgements The support by CAPES and FAPESP is gratefully acknowledged.

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Incidence and resistance profile of *Cedecea sp*. isolated from a hospital

Marcelo M. Antunes[1,2,3,*]; Adriana A. Antunes[4,5]; Galba M. Campos – Takaki[5]

[1] Laboratório Central de Saúde Pública - LACEN, Recife/Brazil;
[2] Hospital Barão de Lucena, Recife/Brazil;
[3] Universidade Federal Rural de Pernambuco - UFRPE, Recife/Brazil;
[4] Pós-Graduação em Ciências Biológicas – UFPE, Recife/Brazil;
[5] Núcleo de Pesquisa em Ciências Ambientais (NPCIAMB) - UNICAP, Recife/PE, Brazil; Rua Fernandes Vieira, S/N, Boa Vista, Recife/PE/Brazil.

[*]Corresponding author: adri_antunes@yahoo.com.br / mantunes@elogica.com.br

The isolation of *Cedecea* in hospitals has been each more frequent, what it represents a significant importance in the determination of the etiology of these infections. A total of 52 *Cedecea* samples were collected from patients in Recife, Brazil, from March 2000 to June 2003. For the bacterial identification and determination of the sensitivity profile to the antimicrobials automated methodology from MicroScan was used, with panels neg. combo, according to the manufacturer's instructions. In a total of 52 isolated samples, 27 were identified as *Cedecea lapagei* (52%), 19 as *Cedecea davisae* (36%) and 6 as *Cedecea* sp. 5 (12%). The sensitivity profile of the isolates is presented, demonstrating a clear tendency to multi-resistance, with higher sensitivity for Fluoroquinolones and Carbapenems. It is possible to conclude that the incidence of *Cedecea*, what in our country is already real, corresponds to less than 1% of all registered infectious processes. The sensitivity profile presented few options to be used as treatment, being necessary not only to standardize the sensitivity tests, as well as to improve the studies in this area in order to achieve an efficient control of this pathogen in the hospital environment.

Keywords *Cedecea sp.*, profile of resistance, antimicrobial tests

1. Introduction

Cedecea is a Gram negative bacillus, belonging to the Enterobacteriaceae family, formerly classified like CDC, enteric group 15, comprising 5 species [1, 2, 3, 4]. It is an important cause of infectious processes, with increased relevance in the hospital environment due to its antimicrobial resistance pattern. The genus *Cedecea* was proposed in 1980 and was formally published in 1981 [5]. *Cedecea davisae* and C. *lapagei* were named in 1981 [5, 6, 7], and *C. neteri* was named in 1982 [2]. In addition, there are two unnamed species; *Cedecea* sp. 3 and *Cedecea* sp. 5 [5, 6, 7]. All five species were defined on the basis of differences in phenotypic properties and DNA hybridization [5, 6, 7]. *Cedecea* is phenotypically distinct from other genera in the *Enterobacteriaceae* family. Cultures are usually lipase positive (corn oil) and resistant to colistin and cephalothin [5, 6, 7]. Those properties are also shared with *Serratia*, but *Cedecea* differs for being unable to hydrolyze gelatin or DNA. *Cedecea* has also been included in some of the commercial identification kits. Among the main sites of infection we can find the respiratory tract, the surgical area, the cardiovascular and urinary systems and osteoarticular points, along with widespread processes such as bacteriemia and septicemia [4]. The isolation of *Cedecea* from hospitals has been more and more frequent, being of significant relevance in the etiology of those infections. There are few studies involving isolated *Cedecea* in Brazilian hospitals.

Objectives

The present work's main objective is to evaluate the presence of *Cedecea* in the hospital environment as an etiologic agent, offering a valuable contribution to the study of the infectious processes originated from this bacterium.

2. Materials and methods

Microorganism

A total of 52 *Cedecea* samples recovered from patients receiving medical care in a public hospital located in the city of Recife, Pernambuco state, Brazil, were collected from March 2000 to June 2003. Reference strains were used as controls.

Isolation and identification

For the isolation, the methodology applied used Blood Agar Base and EMB Agar, incubated for 24 hours at 37ºC. For the sample identification was used the conventional methodology including the following biochemical tests: Glucose, Sucrose, Sorbitol, Raffinose, Rhamnose, Arabinose, Sorbitol, Adonitol, Melibiose, Urea, H_2S production, Indole production, Lysine decarboxylase, Arginine dihydrolase, Ornithine, Trip-deaminase, Esculin hydrolysis, VP, Citrate, Malonate utilization, ONPG, Cl, Cf and Oxidase (Table 1). For the determination of the sensitivity profile to the antimicrobials automated methodology from MicroScan (Dade Behring) was used, with panels neg. combo, according to the manufacturer's instructions, testing the following drugs: Amikacin, Amp-Sulbactam, Ampicillin, Aztreonam, Cephalothin, Cefepime, Ceftazidime, Ceftriaxone, Ciprofloxacin, Gentamicin, Imipenem, Pip-Tazobactam and Sulfa-Trimethoprim (Table 2).

Microscan panels

For *Cedecea* detection the sensitivity test was carried out by microdilution, using an automated methodology from MicroScan, with panels Neg. Combo. Reformulated conventional (Neg. Combo Type 6) panels were inoculated, incubated, and read according to the manufacturer's directions, except for the total incubation time for the conventional panels that was 48 h. Both panel types were incubated in and interpreted with the MicroScan WalkAway-96 system (Dade Behring). Rapid panels were read at 8, 11, and 15h; the results were reported when growth in the control wells was acceptable. Conventional panels were read with the WalkAway-96 automated system after 18 h and then examined visually. The minimal inhibitory concentration (MIC) was determined by microdilution method with Microscan panels Neg. Combo, CLSI cutoff points were used to interpret MIC data (Figure 1).

Figure 1: Following steps of *Cedecea* isolation, identification and interpretation with MicroScan WalkAway-96 automated system (Dade Behring).

3. Results

In a total of 52 samples of *Cedecea*, with 13 being from the Medical Clinic patients (25%), 10 from the Surgical Clinic patients (20%), 8 from the Intensive Care Unit patients (15%), 6 from the Neonatology patients (12%), 5 from Pediatrics patients (9%), 4 from Clinic patients (7%), 3 from Gynecology patients (6%), 2 from Obstetrician patients (4%) and 1 from blood dialysis patients (2%) (Figure 2). In a total of 52 isolated samples, 27 were identified as *Cedecea lapagei* (52%), 19 as *Cedecea davisae* (36%) and 6 as *Cedecea* sp. 5 (12%) (Figure 3). From clinical material, 26 samples were isolated from urine (50%), 10 samples from blood culture (20%), 8 from veined catheter (15%), 6 from secretion of surgical wound (11%), 1 from abscess material (2%) and 1 from LCR (2%) (Figure 4). The sensitivity profile of the isolates is presented, demonstrating a clear tendency to multi-resistance, with higher sensitivity for Fluoroquinolones and Carbapenems (Table 2).

Table 1: Biochemical Characteristics for identification of *Cedece*a species.

Biochemical profile	***Cedecea davisae*** **(19 strains)**	***Cedecea lapagei*** **(27 strains)**
Glucose	+	+
Sucrose	-	-
Sorbitol	-	-
Raffinose	-	-
Rhamnose	-	-
Arabinose	-	-
Inositol	-	-
Adonitol	-	-
Melibiose	-	-
Urea	-	-
H_2S production	-	-
Índole production	-	-
Lysine decarboxylase	-	-
Arginine dihydrolase	+	-
Ornithine	-	-
T. deaminase	+	-
Esculin hydrolysis	+	+
VP test	+	+
Citrate	+	+
Malonate utilization	-	+
ONPG	-	-
Cl	+	+
Cf	+	+
Oxidase	-	-

Table 2: Profile of antimicrobial sensibility in 52 samples of *Cedecea f*rom 1998 to 2003 in Pernambuco, Brazil.

Antimicrobial **Number of strains**	***C. lapagei*** **27**	***C. davisae*** **19**	***C*. sp. 5** **6**
Amikacin	11 %	56 %	0 %
Amp-Sulbactam	15 %	26 %	100 %
Ampicillin	4 %	5 %	17 %
Aztreonam	0 %	33 %	0 %
Cephalothin	0 %	0 %	0 %
Cefepime	6 %	25 %	0 %
Ceftazidime	4 %	42 %	0 %
Ceftriaxone	15 %	32 %	50 %
Ciprofloxacin	48 %	68 %	50 %
Gentamicin	22 %	47 %	0 %
Imipenem	95 %	95 %	90 %
Pip-Tazobactam	44 %	67 %	75 %
Sulfa-Trimethoprim	22 %	42 %	83 %

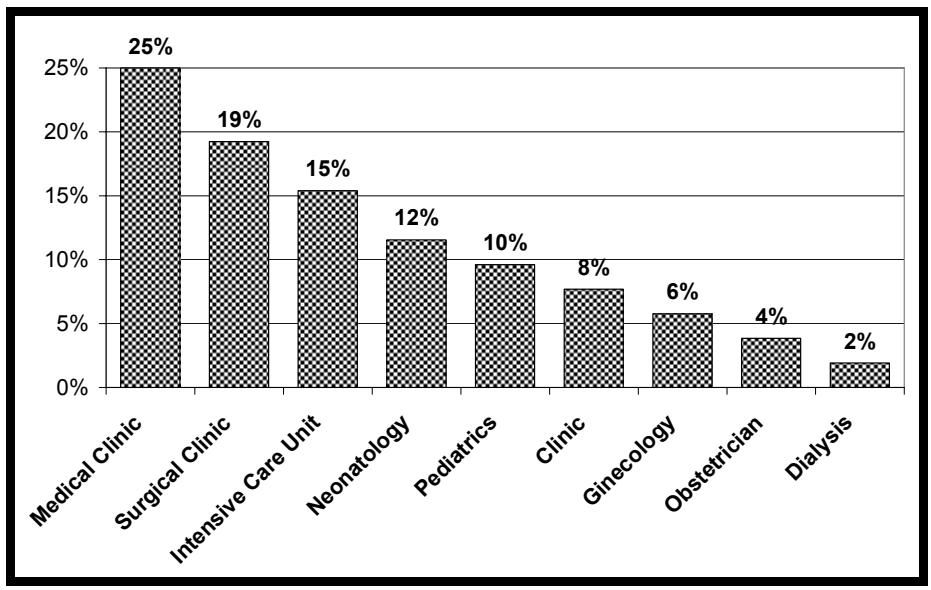

Figure 2: Results of *Cedecea* samples found in hospital environment from 1998 to 2003 in Pernambuco, Brazil.

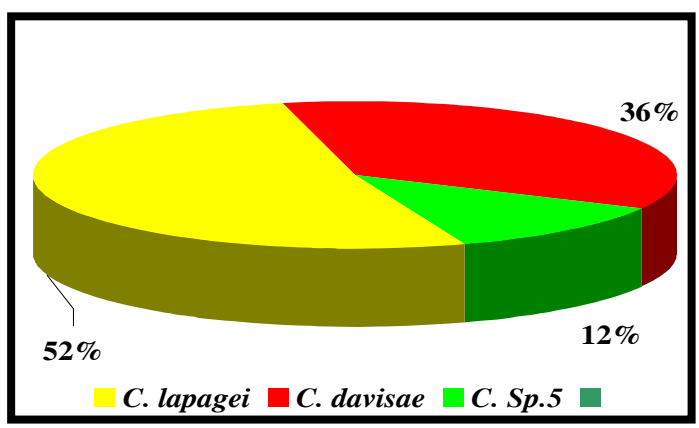

Figure 3: Species of *Cedecea* found in hospital environment from 1998 to 2003 in Pernambuco, Brazil.

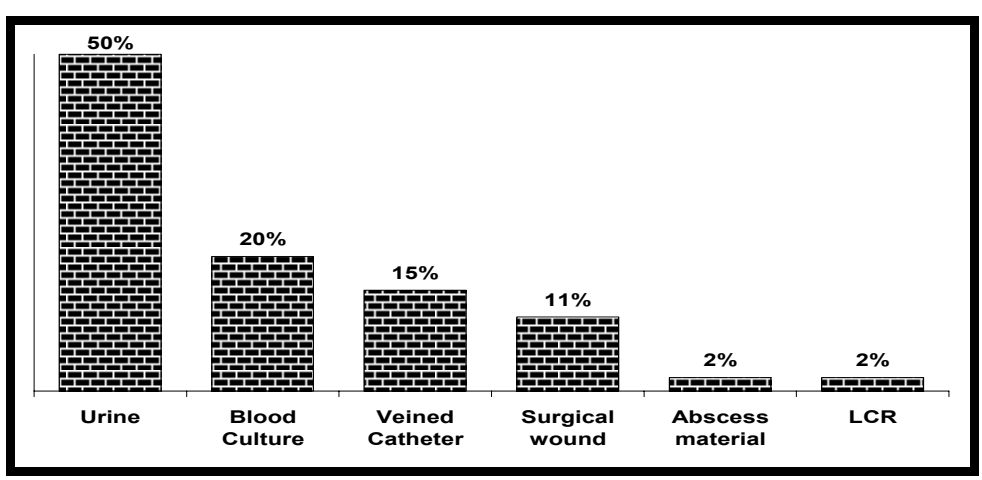

Figure 4: Clinic material found in 52 *Cedecea* samples from 1998 to 2003 in Pernambuco, Brazil.

4. Conclusions

According to the results obtained in the present study, it is possible to conclude that the incidence of *Cedecea*, what in our country is already real, corresponds to less than 1% of all registered infectious processes. The sensitivity profile presented few options to be used as treatment, being necessary not only to standardize the sensitivity tests, as well as to improve the studies in this area in order to achieve an efficient control of this pathogen in the hospital environment.

Acknowledgements This work was carried out with financial support by Dade Behring, CAPES and CNPq.

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Inhibition by doxorubicin of anti-ROS enzymes superoxide dismutase and catalase in *Salmonella typhimurium*

S. Khavari-Nejad[1], **E. Keyhani**[1,2], and **J. Keyhani**[*,2]

[1] Institute of Biochemistry and Biophysics, University of Tehran, 13145 Tehran, Iran
[2] Laboratory for Life Sciences, Saadat Abade, Sarve Sharghi 58, 19979 Tehran, Iran

[*]Corresponding author: e-mail: keyhanius2002@yahoo.com

Doxorubicin (DOXO), an anthracycline antibiotic, is a potent anticancer agent with severe toxic side-effects that have been attributed to its ability to generate free radicals (ROS), particularly in mitochondria. In this study, a prokaryotic organism, *Salmonella typhimurium*, was used to evaluate the effect of the drug on ROS-scavenging enzymes, catalase (CAT) and superoxide dismutase (SOD) activity, and on cell growth and viability. Increasing DOXO concentrations in the culture medium led to increasingly prolonged lag period, reduced growth rate and cell viability, and inhibition of SOD and CAT activity. SOD activity dropped to 66% of the control value in cells grown with 1 μg/ml DOXO and to 34% and 28% in cells grown with 150 and 300 μg/ml DOXO. CAT activity decreased progressively from 93% in 1 μg/ml DOXO to 30% in 150 μg/ml DOXO and was undetectable in 300 μg/ml. Growth in the presence of DOXO led to alterations in cytokinesis, as seen upon examination of cells under fluorescence microscope.

Keywords growth curve; viability; catalase; superoxide dismutase; doxorubicin; *Salmonella typhimurium*

1. Introduction

Isolated nearly forty years ago from *Streptomyces peucetius*, doxorubicin (or adriamycin, DOXO) has been very useful in the clinical treatment of a wide range of tumors such as sarcoma, carcinomas, lymphoma and childhood leukaemia [1]. However its clinical use is limited because of severe toxic side-effects that include cumulative and dose-dependent cardiotoxicity [2]. DOXO efficiency is attributed to its intercalative type of binding with DNA. Its toxicity has been attributed to various factors such as free radicals formation [3, 4], mitochondrial dysfunction [5-7], plasma membrane alteration [8], and suppression of gene expression of cytochrome *c* oxidase II [9]. The general consensus is that DOXO toxicity is linked primarily to its ability to generate free radicals, particularly in mitochondria, with ensuing damage to all cellular organelles [3, 4, 10]. Upon metabolic reduction of DOXO, a semiquinone free radical is produced. Under aerobic conditions, this semiquinone free radical readily produces superoxide anion ($O_2^{\cdot-}$) and, eventually, hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). ROS-scavenging enzymes such as superoxide dismutase (SOD) have been shown to provide some protection against DOXO-induced cardiomyopathy [11]. However, the effect of DOXO on ROS-scavenging enzymes activity remains largely unknown.

The purpose of this research was to evaluate the effect of DOXO on the activity of two anti-ROS enzymes, SOD and catalase (CAT), as well as on the growth curve, cell viability and morphology in a prokaryotic organism, *Salmonella typhimurium*.

2. Materials and Methods

S. typhimurium, strain 3507 [12] was grown at 37°C in a rotary shaker (at 130 rpm), in liquid enriched medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing increasing DOXO concentrations (1, 5, 10, 20, 50, 100, 150, 200, 250 and 300 μg/ml). Aliquots were removed at timed intervals to establish the growth curve and measure cell viability. Cells were harvested after 24 h and washed twice in phosphate buffer (0.01 M, pH 7.0) to determine other parameters (cell morphology, SOD and CAT activity).

Cells grown for 24 h in the presence of increasing DOXO concentrations were examined under a fluorescence microscope (Zeiss - Axioskop 2 Plus) after staining with ethidium bromide.

CAT activity was assayed in cell suspensions by following spectrophotometrically, at 240 nm, the dismutation of H_2O_2; an extinction coefficient of 27 $M^{-1}cm^{-1}$ was used for H_2O_2 at 240 nm [13]. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol H_2O_2 per minute. SOD activity was assayed in lysozyme-treated cells prepared as described in reference [12]; the assay was based on the inhibition of pyrogallol autoxidation in alkaline solution as described in reference [14]. One unit of SOD activity corresponded to the amount required to inhibit pyrogallol autoxidation by 50%. Enzymatic activities were measured in units per mg protein. Protein concentration in samples was determined by the Biuret method.

Electrophoretic separation of proteins in lysozyme-treated cells was performed in 10% non-denaturing polyacrylamide gel, either at 100 V for 5 h, or at 60 V for 18 h. Immediately after electrophoresis, gels were stained either for SOD or for CAT activity. Staining for SOD activity was done in the presence of N,N,N',N'-tetramethylethylendiamine (TEMED), riboflavin and nitro blue tetrazolium, as described in reference [15]; staining for CAT activity was performed in the presence of horseradish peroxidase as described in reference [13], except that o-dianisidine was used as the reducing substrate.

3. Results

3.1 Effect of doxorubicin on cell growth

Figure 1 shows the growth curves obtained by monitoring the increase in cell concentration, measured at timed intervals by direct microscope count, for *S. typhimurium* cultured in the presence of increasing DOXO concentrations. As the concentration of DOXO in the culture medium increased, the duration of the lag phase and of the log phase progressively increased while the growth rate progressively decreased; growth was negligible in 250 μg/ml DOXO.

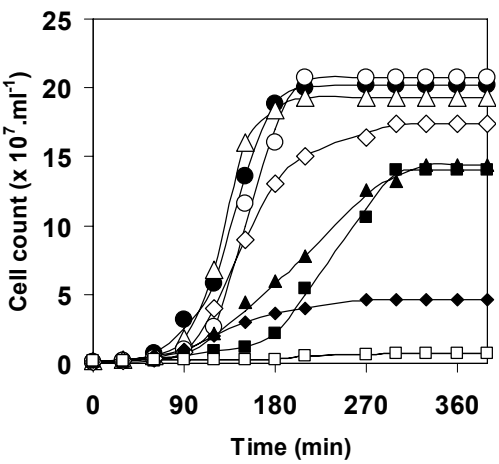

Fig. 1 Growth curves of *S. typhimurium* cultured at 37°C in enriched medium (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.0) in the presence of increasing DOXO concentrations (● control, △ 1, ○ 5, ◇ 10, ▲ 20, ■ 100, ◆ 200, □ 250 μg/ml DOXO)

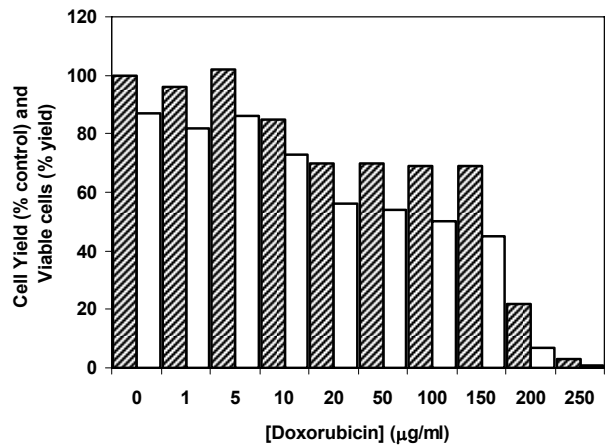

Fig. 2 Cell yield (▨), expressed as % control, and cell viability (□), expressed as % of the yield, after 6 h growth in the presence of increasing DOXO concentrations.

Examination of the cells under a fluorescence microscope showed that, after 24 h culture in the presence of DOXO concentrations of 50 μg/ml and above, cells remained occasionally attached to each other, forming short filaments. Similar observations were made for *Candida utilis* cultured in the presence of DOXO [16].

3.2 Effect of doxorubicin on cell viability

The cell yield and the percentage of viable cells obtained after 6 h culture in the presence of increasing concentrations of DOXO are shown in Figure 2. The yield dropped by 15% in 10 μg/ml DOXO and by 30%, 78% and 97% in 20, 200 and 250 μg/ml DOXO, respectively. The percentage of viable cells remained at the control value, approximately 90%, in up to 10 μg/ml DOXO. As DOXO concentration increased from 10 to 150 μg/ml, the percentage of viable cell dropped progressively from 86% to 65%. When DOXO concentration reached 200 and 250 μg/ml, the percentage of viable cells dropped steeply to 33% and 23%, respectively (Fig. 2).

3.3 Effect of doxorubicin on SOD and CAT activities

Figure 3a shows the variation in CAT activity detectable in cells cultured for 24 h in the presence of increasing DOXO concentrations. The activity decreased progressively to 50% of the control value in 50 μg/ml DOXO and to undetectable levels in 300 μg/ml DOXO. For SOD (Fig. 3b), there was also a DOXO concentration-

dependent decrease in activity with 50% of the control value detectable in 50 μg/ml DOXO. However, in contrast with CAT, SOD activity dropped abruptly to 66% in 1 μg/ml DOXO while 30% of SOD activity was still detectable in 300 μg/ml DOXO.

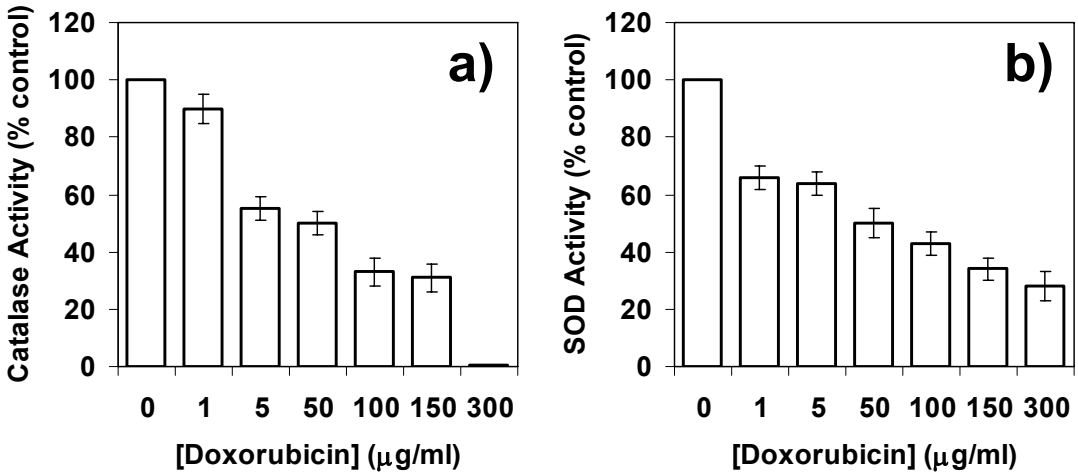

Fig. 3 a) CAT activity and b) SOD activity detectable in *S. typhimurium* cultured for 24 h in the presence of increasing DOXO concentrations. The activities, expressed as % control, were measured in units/mg protein.

Results were confirmed when *S. typhimurium* cells cultured in the presence of increasing DOXO concentrations were electrophoresed in 10% polyacrylamide gel under non-denaturing conditions, after treatment of the cells with lysozyme. Following activity staining for CAT, one band was detectable in the control sample (lane 1, Fig. 4 a) and in samples grown in the presence of 1-50 μg/ml DOXO (lanes 2-5, Fig. 4 a); the band, corresponding to a CAT of estimated molecular weight 360 kD, was barely detectable in samples grown in the presence of 100-150 μg/ml DOXO (lanes 6-7, Fig. 4a) and no band was detectable in samples grown in the presence of 300 μg/ml DOXO (lane 8, Fig. 4 a).

Following activity staining for SOD, three bands were revealed, corresponding to SODs of estimated molecular weight 39 kD, 33 kD, and 28 kD, respectively (Fig. 4b). The high (39 kD) and low (28 kD) molecular weight SODs were detectable in all samples, but with decreasing intensity as DOXO concentration increased (lanes 1-8, Fig. 4b). The intermediary size (33 kD) SOD was not detectable in the control (lane 1, Fig. 4b) but was present with increasing intensity in samples grown in the presence of 1-50 μg/ml DOXO (lanes 2-5, Fig. 4b). It was still detectable, but with decreasing intensity, in samples grown in the presence of 100-150 μg/ml DOXO (lanes 6-7, Fig. 4b), and it was undetectable in sample grown in the presence of 300 μg/ml DOXO (lane 8, Fig. 4b).

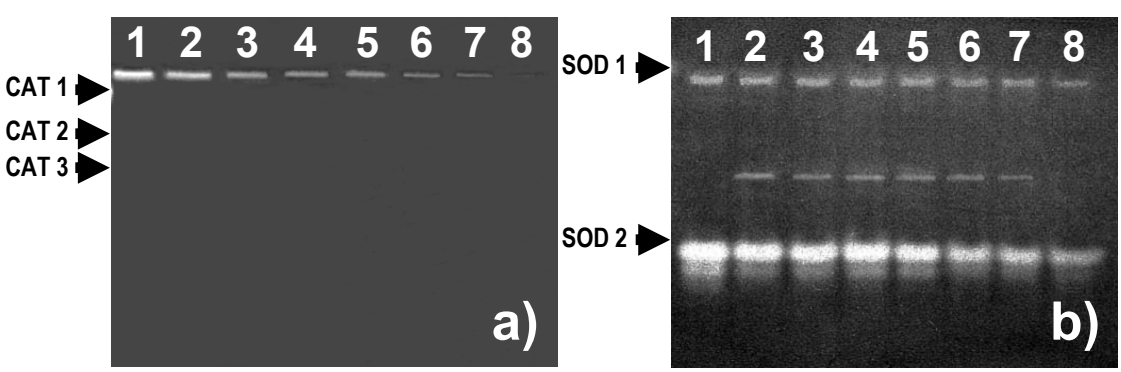

Fig. 4 Non-denaturing polyacrylamide gel electrophoresis of *S. typhimurium* proteins stained a) for CAT activity or b) for SOD activity. Gel a) - lanes 1-8: 60 μg protein of *S. typhimurium* grown for 24 h in the absence (lane 1) and in the presence of, respectively, 1, 5, 20, 50, 100, 150 and 300 μg/ml DOXO (lanes 2-8). Arrows indicate migration of *Aspergillus niger* CAT (354 kD) (CAT 1), beef liver CAT (250 kD) (CAT 2), guinea pig liver CAT (238 kD) (CAT 3); Gel b) – lanes 1-8: 30 μg protein of *S. typhimurium* grown for 24 h in the absence (lane 1) and in the presence of, respectively, 1, 5, 20, 50, 100, 150 and 300 μg/ml DOXO (lanes 2-8). Arrows indicate migration of *E. coli* MnSOD (40 kD) (SOD 1) and *E. coli* FeSOD (28 kD) (SOD 2).

4. Discussion

The mechanisms of action of DOXO, one of the most potent anticancer drug, are still the subject of controversy, in particular those responsible for the drug toxic side-effects. Diverse sites of action such as mitochondria [5-7], plasma membrane [8], etc., have been proposed. Currently, the most important cause of DOXO toxic effects is considered to be the formation of ROS [3-5]. Indeed, elevation in the intracellular ROS concentration renders cells highly susceptible to cell death [10], even though ROS such as superoxide anion and hydrogen peroxide, normal products of cellular metabolism, are indispensable in low concentrations for signal transduction pathways and redox state of the cell [17]. It has been reported that anti-ROS such as CAT and SOD decreased apoptosis induced by DOXO [2, 11, 18, 19]. CAT is an important antioxidant enzyme, which has been shown to provide cardiac protection from toxicity induced by DOXO [2]. In CAT overexpressing transgenic mice, catalase elevation in the heart prevented DOXO chronic cardiomyopathy [11]. SOD possesses a large therapeutic value against ROS [18]. However, SOD, CAT were found to be significantly reduced by DOXO-vincristine-dexamethazone therapy [19].

Thus, in *S. typhimurium*, the reduction in the activity of CAT and SOD reported in this work reduced the ability of the bacterium to fight the overproduction of ROS by DOXO and enhanced the drug adverse effects. Consequently, DOXO caused a wide range of alterations in *S. typhimurium* including dysfunction in the cell division, alteration of cytokinesis and formation of filaments of cells still attached to one another. Interestingly, although the overall SOD activity decreased promptly as DOXO concentration increased, a third SOD band, possibly due to Cu-Zn SOD, was detectable by gel electrophoresis in cells grown in the presence of 1-150 µg/ml DOXO. This could be a defense mechanism of the cell against ROS as it has been shown that Cu-Zn SOD plays an important protective role against ROS in various *Salmonella* strains [20, 21]. Eventually it was itself hampered by increasing DOXO concentrations. Finally, one should keep in mind that DOXO therapeutic role is essentially attributed to its interaction with DNA. This itself entails potentially multiple effects on the cell physiology, including protein synthesis and its regulation. Thus, while DOXO could have a direct inhibitory effect on anti-ROS enzymes activity, it could also impair their synthesis. Increase as well as decrease of SOD activity during DOXO treatment have been reported [18, 22]. However, while anti-ROS enzymes have been shown to protect against DOXO toxic side-effects, they may hamper the oxystress cancer therapy that has been proposed based on the ROS-dependent activation of apoptotic cell death [17].

Acknowledgements Suported by the J. and E. Research Foundation, Tehran, Iran.

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Secondary metabolites produced by endophytic fungus *Paecilomyces variotii* Bainier with antimicrobial activity against *Enterococcus faecalis*

M. R. Oliveira Silva[*,1,2], **K. Xisto Sena**[2] and **N. Buarque Gusmão**[2]

[1] PhD Student in Biology of Fungi, Department of Mycology, Federal University of Pernambuco, Av. Prof. Morais Rego, 1235, 50670-901, Recife, Pernambuco, Brazil

[2] Laboratory of Antimicrobial Assays, Department of Antibiotics, Federal University of Pernambuco, Av. Prof. Morais Rego, 1235, 50670-901, Recife, Pernambuco, Brazil

[*]Corresponding author: e-mail: mroseosilva@hotmail.com, Phone: +55 81 21268346

Enterococcus faecalis is a Gram-positive bacteria considered an important emergent nosocomial pathogen, mainly due to its resistance to most antibiotics and responsible for a variety of human infections. In order to discover new bioactive secondary metabolites from microbial sources, the objective of this work was to evaluate the antimicrobial activity of the crude extract obtained from *Paecilomyces variotii* for eight clinical isolates of *E. faecalis* by the disk diffusion method. The results showed that all clinical isolates were inhibited by fungal extract, with inhibition zones ranging from 25 to 35.25 mm. In addition, the results also revealed resistance, in the microorganisms test, to antibiotics such as ciprofloxacin, cotrimoxazole, erythromycin, gentamicin, oxacillin and tetracycline.

Keywords Antimicrobial Activity; *Paecilomyces variotii*; Endophytic Fungi; *Enterococcus faecalis*

1. Introduction

Enterococci are usual inhabitants in the human intestinal microbiota, mainly in the large intestine and genital-urinary tract, and generally they are not regarded as pathogenic organisms. However, in literature it has been cited that enterococci are emerging as prominent nosocomial pathogens, being responsible for a variety of human ailments [1, 2]. *Enterococcus faecalis* is a member of the genus *Enterococcus* considered the major opportunistic pathogen isolated from hospital infections, with incidence of 80-90% [3]. The survival of this microorganism in the hospital environment is due to the elevated level of acquired resistance to conventional antibiotics, such as aminoglycosides and glycopeptides, but its resistance to ampicillin is still rare [4].

The increased in drug-resistance by bacteria is considered a serious problem for public health, constituting a challenge for researchers worldwide to search for new antimicrobials agents. Nowadays, research groups have focused interest on microorganisms obtained from different ecosystems, probably due to their metabolic pathway diversity in consequence of the selective environmental conditions. Endophytic fungi are recognized as useful sources of secondary metabolites because depending on the biotype, ecological niche and host plants, these microorganisms are able to produce appropriate bioactive compounds [5, 6]. There are several reports on antimicrobial compounds produced by this specific group of fungi [7-10].

In an attempt to discover a novel substance of medicinal importance, the present work aims to evaluate the antimicrobial activity of the secondary metabolites obtained from *Paecilomyces variotii*, an endophytic fungus isolated from *Laguncularia racemosa* mangrove plant.

2. Material and Methods

2.1 Endophytic fungus

Paecilomyces variotii strain was isolated from the leaves of *Laguncularia racemosa* (L.) Gaertn. (White mangrove, Combretaceae) collected from estuary of the Paripe River, Ilha de Itamaracá, Pernambuco, Brazil. The fungal identification was performed by observing macroscopic and microscopic characteristics such as colour, colony morphology and conidial size. The fungus was maintained on potato dextrose agar (PDA) slants at ± 4°C until further use.

2.2 Fermentation

In order to obtain secondary metabolites, the fungal strain was grown in PDA culture medium at 30°C for 3 days. After that, a pre-inoculum was prepared by introducing small fragments (1 cm^2) of the growth culture into 250 mL Erlenmeyer flasks containing 50 mL of Malt broth (1.5% Malt Extract, pH 6.9 ± 0.1) and cultivated on a rotary shaker at 200 rpm, ± 28°C (room temperature) for 3 days. Subsequently, the pre-inoculum was transferred to Fernbach flasks containing 450 mL of the same culture medium and cultivated as described above for 7 days.

2.3 Obtention of crude extract

At the end of fermentation, the culture broth was separated from the mycelium by filtration and the filtrate was extracted with ethyl acetate (1:1, v/v) under constant shaking for 2 hours. The organic phase was concentrated under reduced pressure using a rotary evaporator at ± 45°C and, finally, the concentrated extract was stored in a vacuum desiccator.

2.4 Microorganisms test

For the antimicrobial activity test, eight clinical isolates of *Enterococcus faecalis* were used: 21675, 21752, 21944, 24614, 24622, 24708, 24962 and 25392 (Table 1). All clinical isolates were provided by Laboratorial Unit of the University Hospital, Federal University of Pernambuco, Recife, Brazil. The confirmation of the genus *Enterococcus* was determined by plating in bile-esculin and brain heart infusion plus 6.5% NaCl agars culture media. Sequentially, the bacterial cultures were maintained on growth medium (1% Peptone, 0.3% Beef Extract, 0.5% NaCl, 1% Yeast Extract, 1% Glucose, 1.5% Agar, pH 7.0 ± 0.1) at ± 4°C.

Table 1 *Enterococcus faecalis* isolated from different sources.

Clinical Isolates	Source
21675	Ulcer
21752	Tracheal secretion
21944	Blood
24614	Vaginal secretion
24622	Tracheal secretion
24708	Secretion*
24962	Tissue fragment
25392	Bubble fluid

* Not Specified

2.5 Antimicrobial assay

Antimicrobial activity test was carried out by disk diffusion method [11]. The crude extract was dissolved in dimethylsulfoxide (DMSO) until a final concentration of 50 mg/mL. Sterile paper disks were impregnated with 20 µL of the extract and placed on the Petri dish surfaces containing Mueller-Hinton agar medium previously spread with bacteria suspensions adjusted according to McFarland standard solution 0.5. Then, the Petri dishes were incubated at 35°C and the diameter of the inhibition zones (in mm) was measured after 48 hours. All data were analyzed using Microsoft Excel for Windows.

2.6 Reference antibiotics

The susceptibility profile of each clinical isolate was determined using disks of the following reference antibiotics: ampicillin (10 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg), penicillin G (10 UI), tetracycline (30 µg) and vancomycin (30 µg).

3. Results and Discussion

Fig. 1 shows that all *E. faecalis* clinical isolates were susceptible to the crude extract at 100 µg/disk final concentration, with inhibition diameters ranging from 25 to 35.25 mm. The isolate number 24962 was the most susceptible and presented an expressive result, with inhibition diameter of 35.25 mm, followed by the isolates 24622 and 24614 with inhibition diameters of 29.25 mm and 27.25 mm, respectively. Furthermore, these microorganisms were resistant to the antibiotics erythromycin, gentamicin, oxacillin, tetracycline and cotrimoxazole and the isolate 24614 was even resistant to ciprofloxacin as can be observed in Table 2.

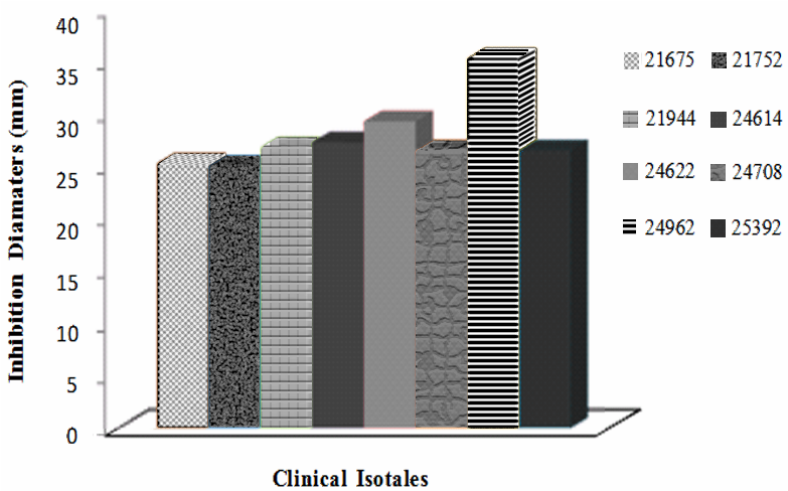

Fig. 1 Antimicrobial activity test using the crude extract (100 µg/disk) obtained from *P. variotii* for *E. faecalis* clinical isolates.

Table 2 Susceptibility test using reference antibiotics.

Clinical Isolates	Antimicrobial Agents								
	Amp	Cipro	Cotri	Ery	Gent	Oxa	Pen G	Tet	Vanc
21675	S	S	R	S	R	R	S	S	S
21752	S	S	R	S	R	R	R	S	S
21944	S	R	R	R	R	R	S	R	S
24614	S	R	R	R	R	R	S	R	S
24622	S	S	R	R	R	R	S	R	S
24708	S	S	R	R	R	R	S	R	S
24962	S	I	R	R	R	R	S	R	S
25392	S	R	R	R	R	R	S	R	S

Amp- Ampicillin, Cipro- Ciprofloxacin, Cotri- Cotrimoxazole, Ery- Erythromycin, Gent- Gentamicin, Oxa- Oxacillin, Pen G- Penicillin G, Tet- Tetracycline and Vanc- Vancomycin.
R- Resistant, I- Intermediate, S- Susceptible

The efficacy of ampicillin and vancomycin was 100% for *E. faecalis*, indicating the success of these antibiotics in infections caused for this pathogen. The elevated incidence of *E. faecalis* isolated from brazilian hospitals is basically due its intrinsic resistance to several antibiotics and also acquired resistance by mutation or/and transferring of genetic material such as plasmids and transposons [12].

Previous studies have demonstrated the potential of producing substances with biological activity by endophytic fungi: *Penicillium janthinellum* from *Melia azedarach* (Meliaceae) [13]; *Penicillium janczewskii* from *Prumnopitys andina* (Podocarpaceae) [14]; *Aspergillus fumigatus* from *Cynodon dactylon* (Poaceae) [15]; *Curvularia* sp. from *Ocotea corymbosa* (Fabaceae) [16]; *Phomopsis* sp. from *Erythrina crista-galli* (Fabaceae), *Garcinia* species, *Aspidosperma tomentosum* (Apocynaceae) and *Spondias mobin* (Anacardiaceae) [17-19]; *Guignardia* sp. from *Spondias mobin* (Anacardiaceae) [20].

The genus *Paecilomyces* presents several species that are able to produce a wide variety of bioactive secondary metabolites with cytotoxic, immunostimulating and antimicrobial activities [21]. Brefeldin A produced by *Paecilomyces* sp. isolated from *Taxus mairei* and *Torreya grandis* has been associated with cytotoxic activity [22]. In another work, *Paecilomyces* sp., an endophytic fungus isolated from medicinal plants, produces secondary metabolites that show promising antitumor and antifungal activities [23].

Recently, research on the secondary metabolites of endophytic fungi from estuarine environment has led to the isolation of many new compounds with different bioactivities. Xyloketal D, G, H are substances isolated from mangrove fungus *Xylaria* sp. [24-26]. Two unidentified fungi isolated from *Kandelia candel* (Rhizophoraceae) and *Avicennia marina* (Avicenniaceae) are able to produce metabolites with antitumor and cytotoxic activities, respectively [27, 28]. Avicennin A and B are compounds obtained from the mangrove fungus of *Avicennia marina* (Avicenniaceae) and *Penicillium thomi* isolated from *Bruguiera gymnorrhiza* (Rhizophoraceae) which produces metabolites recognized for antitumor activity [29, 30]. In addition, paecilin A and B are two new chromone derivates from mangrove endophytic fungus *Paecilomyces* sp. [31]. The antimicrobial potential of the mangrove endophytic fungi is also very well documented in literature [32].

4. Conclusions

Our results suggest that mangrove endophytic fungi are gaining importance because of their enormous potential to produce novel bioactive compounds of medicinal importance. Further purification and characterization of the fungal extract reported in this study may lead to the discovery of novel antimicrobial compounds of pharmaceutical importance, which could then be produced on a large scale.

Acknowledgements The authors are grateful to Professor Thomas Henry Greenhalgh for english review and CAPES for financial support.

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Serum as an environment to live or not to live for Gram-negative bacteria: relationship between lysozyme and complement system in killing *Salmonella* O48 strain

G. Bugla-Ploskonska[*,1], A. Kiersnowski[2], B. Futoma-Koloch[1], and W. Doroszkiewicz[1]

[1] Department of Microbiology, Przybyszewskiego 63/77, Institute of Genetics and Microbiology, University of Wroclaw, 51-148 Wroclaw, Poland

[2] Polymer Engineering & Technology Division, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland

[*]Corresponding author: e-mail: gabi@microb.uni.wroc.pl

The bactericidal effect of serum plays a key role in humoral defense against microbial pathogens. Lysozyme - cooperates with the complement system in the bactericidal action of serum. Serotype O48 *Salmonella* belongs to clinically important bacteria causing diarrhoea in infants and children. Our present results demonstrated that the most efficient killing of *Salmonella* O48 occurred when all components of normal bovine serum (NBS) cooperated with each other. It is very interesting that elimination of lysozyme from NBS by using bentonite significantly decreased the bactericidal activity of NBS against *Salmonella* O48 strain. The results of X-ray diffractometric studies suggested that apart from lysozyme, other components of serum were adsorbed on the bentonite particles.

Keywords serum; lysozyme; *Salmonella*; bentonite (montmorillonite)

1. Introduction

The complement systems of higher animals comprises a group of more than 30 soluble proteins and receptors that play an important role in innate and acquired immunity the host defence mechanisms against infection, and participate in various immunoregulatory processes [1]. Sensitivity of bacteria to the bactericidal activity of complement depends on the structure and organisation of bacterial outer membrane. Rough strains (R) of Gram-negative bacteria that have LPS lacking of an O - antigenic polysaccharide chain are generally susceptible to the bactericidal action of serum in contrast to smooth (S), resistant strains [2, 3]. The LPS of *Salmonella* O48 that we tested was S type [4]. In the bactericidal action of serum with complement cooperates the lysozyme, the enzyme that catalyses the hydrolysis of β 1,4 linkage between *N*-acetyloglucosamine and *N*-acetylmuramic acid in the bacterial cell wall [5]. The role of lysozyme in the bactericidal action of serum is controversial, but many Gram-negative bacteria are killed after exposure to lysozyme - free serum by a process that requires participation of complement system [6]. *Salmonella enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates and are usually transmitted by food or water contaminated by infected faeces. Human infections with serovars of *Salmonella salamae, bongori* and *diarizonae* are the mainly results of contact with reptiles [7]. Our previous experimental results [8] indicated that within the serotype *Salmonella* O48 was the strains sensitive to the bactericidal action of normal bovine serum (NBS). The scope of this work is a comparison the susceptibility of this sensitive strain to the NBS devoid of lysozyme. The aim of structural research was to trace transitions accompanying adsorption of serum proteins on the particles of montmorillonite.

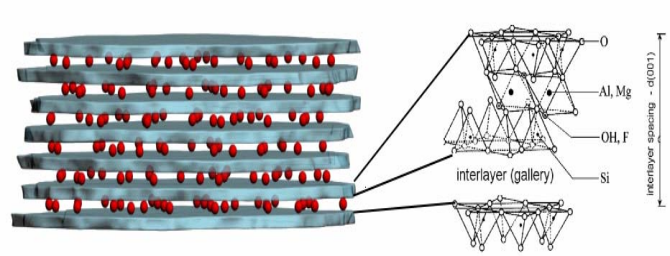

Fig.1 Montmorillonite is a clay silicate with remarkable structure consisting of two tetrahedral layers "sandwiching" single layer of octahedral structural units. In montmorillonites, aluminum ions (Al^{3+}) placed in octahedral gaps, are partially exchanged by magnesium (Mg^{2+}) or potassium cations (K+), which cause a weak negative charge of the single sheet.

In the environment, montmorillonite occurs as a component of the mineral called bentonite – a kind to interstratified illite-smectite clay minerals that are ubiquitous in sedimentary basins. Due to its sorption

properties it is often applied as decontaminating agent for wastewaters, selective material for ions uptake or, as in this work as a mineral for selective removal of proteins from natural systems [9, 10, 11].

2. Materials and Methods

2.1 Bacterial strains

The study was carried out on 10 strains of serotype *Salmonella* O48. The complete list of tested strains is presented in Table 1.

Table 1 The origin and antigenic characteristics of *Salmonella* O48 strains used in this study

Species	Subspecies	Serovar	Antygen O48	Source	
Salmonella enterica	*enterica*	Dahlem	k:e:n:z_{15}	PCM 2512	KOS 1166
		Djakarta	z_4,z_{24}:-	PCM 2513	KOS 432
		Hisingen	a:1,5,7	PCM 2536	NBIMCC 1357
		Toucra	z:1,5,/z_{58}/	PCM 2515	KOS 1386
		Isaszeg	z_{10}:e,n,x	PCM 2550	IP 886/71
		Fitzroy	e,h:1,5	PCM 2549	IP 407/68
	salamae	Erlangen	g,m,b:-	PCM 2533	NBIMCC 17259
		Sakaraha	k:z_{39}	PCM 2538	NBIMCC 1287
	diarizonae		r:e,n,x,z_{15}	PCM 2511	KOS 1594
S. bongori		Bongori	z_{35}:-	PCM 2547	CNCTC Sk 2R/68

2.2 Serum

Normal bovine serum (NBS) was obtained from five healthy animals, which were not subjected to any antimicrobial drug treatment. The serum samples were collected, pooled and kept frozen (-70°C) for a period no longer than three months. The suitable volume of serum was thawed immediately before use.

2.3 Bentonite (montmorillonite, MMT) - adsorbed serum

We used catalytic grade sodium montmorillonite purchased from Sigma. Lysozyme removal from NBS was accomplished as described previously [9, 12]. This MMT - adsorbed NSB was used in the experiments concerning the bactericidal action of NBS. Afterwards pelletized samples were used WAXS measurements.

2.4 Normal bovine serum (NBS) bactericidal assay

The bactericidal activity of NBS was determined as described previously [13]. The number of colony forming units (CFU) at time 0 was taken as 100%. Strains with survival rate in 50% serum after 180 min. of incubation >100% were considered resistant, strains with survival rate <100% were considered susceptible to bactericidal action of serum. NBS decomplemented by heating the sample at 56°C for 30 min (NBS 56°C) was used as the control.

2.5 Structural investigations-adsorption of the proteins on montmorillonite [14]

Wide- and small angle X-ray diffraction. **WAXS** (wide angle X-ray scattering) experiments were performed on a SEIFERT URD6 diffractometer with Ni-filtered CuKα radiation generated by sealed X-ray tube. The radiation source was powered by a high-voltage generator operated at 40 kV and 30 mA. Data was collected in a step-scan mode (in 0,1°/2θ steps) within the range of 2θ from 1° to 15° that gives s-vector ranging from 0,11 nm-1 to 2,25 nm-1 ($s=2\sin\theta/\lambda$).

3. Results

Our obtained results concerning the sensitivity of *Salmonella* O48 strains to NBS-MMT (serum treated with bentonite – MMT) are given in Tables 2.

Table 2 Bactericidal activity of NBS-MMT (serum treated with bentonite – MMT, to remove lysozyme)

Salmonella O48 strain no	50 % NBS-MMT				50% NBS	
	CFU[1]				% of survival of bacterial cells in serum after 3h of incubation	
	T0[2]	T1 (after 60 min)	T3 (after 180 min)	% of survival of bacterial cells in serum after 3h of incubation	Control I[3]	Control II[4]
Djakarta PCM 2513	$24x10^5$	$55x10^4$	$55x10^3$	2,3	0,001	1529,4
Erlangen PCM 2533	$15x10^5$	$16x10^4$	$59x10^4$	39,3	0,03	857,0
Fitzroy PCM 2549	$21x10^5$	$54x10^5$	$26x10^5$	123,8	0,03	1950,0
Sakaraha PCM 2538	$13x10^5$	$13x10^5$	$17x10^5$	130,8	0,7	3722,0
Hisingen PCM 2536	$24x10^5$	$22x10^5$	$34x10^5$	141,7	0,001	2103,4
diarizonae PCM 2511	$12x10^5$	$12x10^5$	$26x10^5$	216,7	0,7	3722,0
Isaszeg PCM 2550	$22x10^5$	$17x10^5$	$62x10^5$	218,8	1,05	714,3
Toucra PCM 2515	$47x10^5$	$68x10^5$	$19x10^6$	404,3	0,2	1527,8
Dahlem PCM 2512	$17x10^5$	$51x10^5$	$31x10^6$	1823,5	0,001	1100,0
Bongori PCM 2547	$18x10^5$	$59x10^5$	$37x10^6$	2055,6	0,09	3600,2

[1]CFU - colony forming units
[2] T0- 100% of survival of bacterial cells in serum
[3]Control I - % of survival of bacterial cells in NBS after 3h of incubation
[4]Control II - % of survival of bacterial cells in NBS decomplemented by heating at 56°C for 30min

Two serovars of *Salmonella* O48 were sensitive to the bactericidal effect of bovine complement in which the lysozyme was removed. Eight strains demonstrated higher resistance to the bactericidal activity of NBS-MMT. After treating the serum with MMT, the ratio of percentage survival of bacterial cells was above 123,8 to 2055,6 after 3 hour of incubation. In these cases the bactericidal activity of NBS was lost after the MMT treatment.

X-ray diffractometric studies

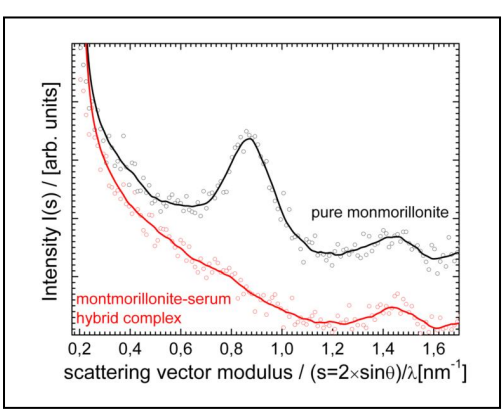

Fig. 2 WAXS traces recorded for unmodified montmorillonite (upper /red/ curve) and the montmorillonite treated with serum (lower /black/ curve)

The powder, wide-angle X-ray diffractogram (WAXS) recorded for neat montmorillonite within 2θ of 1-15° (Fig. 2, upper curve) reveals a discrete maximum at the scattering vector of approximately 0,8 nm^{-1}, which indicates the existence of regular, layered structure of the clay. The interlayer distance (d) calculated according to the Braggs' law (1) from this maximum equaled 1,1 nm.

$$d = \frac{n\lambda}{2\sin\theta_{max}} = \frac{1}{s_{max}} \qquad (1)$$

where:

d – interlayer distance
n – diffraction order (here is always equal to unity)
λ – wavelength of the X-ray beam (here CuK_{α}=0,1542 nm)
θ_{max}, s_{max} – the location of diffraction maximum expressed as half of diffraction angle (2θ) or scattering vector (s).

The WAXS curve recorded for the silicate with adsorbed proteins is featureless (Fig. 2, lower curve), which proves that stratified arrangement of the layered crystal after adsorption no longer exists.

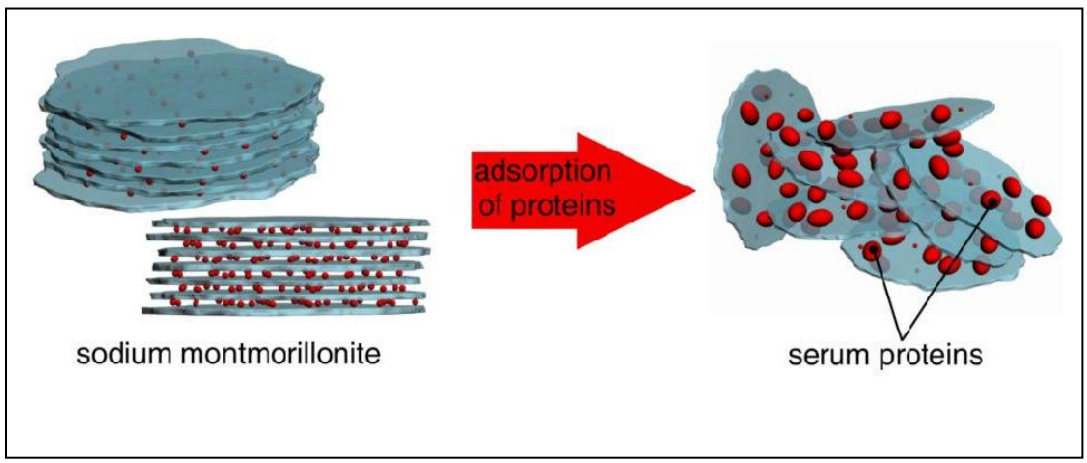

Fig.3 Cartoon representation of structural changes in crystal structure of montmorillonite occurring upon adsorption of proteins from serum

Thus, one can conclude that montmorillonite exfoliates during the adsorption, forming disordered system of statistically oriented silicate platelets according to the simplified model shown in Fig. 3

4. Discussion

Complement-mediated lethal action of serum and phagocytosis are important mechanisms of the host defense against infection with Gram-negative bacteria. The complement system of the animals forms a powerful immune barrier [15]. The role of lysozyme in the bactericidal action of serum is controversial. Donaldson *et al*. [9] has shown that the most efficient killing of Gram-negative bacteria by serum occurred when *β*-lysin, lysozyme and the compelment system acted together at usual serum concentrations. Many authors [16, 17], using sera from which lysozyme has been removed came to a conclusion that the lysozyme was unnecessary in the bacterial process. Other workers reported that the sera from which lysozyme was removed have a weaker bactericidal activity against various Gram-negative rods, as compared to that of full bactericidal serum, and elimination of lysozyme from serum by bantonite absorption affected serum bactericidal activity the least [18, 19].

In our experiment we proved that lysozyme participates as an obligatory factor in the bactericidal action of the components of normal bovine serum for eight tested *Salmonella* serovars. The reduction of the bactericidal activity of NBS after MMT treatment is very interesting. The easiest way to investigate mechanism of the adsorption is monitoring the value of interlayer spacing (d(001)) by means of X-ray diffraction techniques. On the basis of the recorded values and structural behavior, one can estimate the dimensions of molecules adsorbed in the interlayer regions of the silicate and interactions between the molecules and nanoparticles [20, 21, 22]. Since, according to literature [23] adsorption of single proteins of polypeptides does not cause exfoliation, while the second was observed, we assumed that the mechanism of exfoliation relies on synergistic adsorption of several proteins on surface of the silicate. Aramini et al. [24] suggested that lysozyme such as some milk protein, citrates and phosphates is know to have high affinity binding sites for calcium. Calcium ions (Ca^{2+}) are required for classical pathway complement activation, and lysozyme absorbed onto MMT may act as inhibitor of this activation for strains (*S*. Djakarta and Erlangen) which required this pathway complement activation. Bentonite treatment did not dramatically reduce the antibacterial activity of NBS against this strains. Lysozyme from bovine serum absorbed onto MMT was found as a component of serum which inhibit the classical pathway of serum complement activation.

It appears that apart from lysozyme, bovine serum contains another component having crucial influence on bactericidal action of serum and final lysis of the bacterial cells. As revealed in our experiments, this compound

(protein?) is being adsorbed on MMT particles together with lysozyme. Hill et al. [25] suggested that the β-lysin and C1q may be removed (adsorbed on MMT) together with lysozyme. Currently this phenomenon is being investigated at our laboratories.

Acknowledgements The authors thank prof. dr hab. A. Gamian (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland) for the Salmonella strains from the Polish Collection of Microorganisms.

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Towards the eradication of Poliomyelitis

M.J. Pozuelo de Felipe*, F. Llinares Pinel and P. Jiménez Gómez

Departamento de Bioquímica, Biología Molecular y Celular. Sección Microbiología. Facultad de Farmacia. USP CEU. Boadilla del Monte. 28668 Madrid.

*Corresponding author: marpoz@ceu.es

In 1988, the World Health Assembly resolved to eradicate poliomyelitis globally. The number of countries in which polio is endemic declined from 125 to 4 by the end of 2006. Although progress towards interrupting transmission has continued, it is currently endemic in four countries and, on occasion, this goal is threatened by a resurgence of polio caused by the poliovirus spread from endemic countries to previously polio-free countries.
This report describes the general features of poliovirus, the etiologic agent and clinical features of poliomyelitis, resumes the general measures adopted by WHO (World Health Organization) for polio eradication, and the evolution of the incidence to date.

Keywords poliomyelitis, poliovirus, eradication, vaccination.

1- Poliovirus

Poliovirus is a human enterovirus, a member of the family of *Picornaviridae*. The viral particle is about 30 nm in diameter with icosahedral symmetry. Poliovirus is composed of single stranded positive-sense RNA, which is about 7,500 nucleotides long, and a protein capsid composed of four proteins: VP1, VP2, VP3 and VP4**.** It is a non- enveloped, heat, detergent and acid pH resistant virus.

There are three serotypes of poliovirus: PV1 (Mahoney or Brunhilde), PV2 (Lansing), and PV3 (Leon), each with a slightly different capsid protein. Capsid proteins define cellular receptor specificity and virus antigenicity. PV1 is the most common form encountered in nature, although all three forms are extremely infectious [1].

2- Epidemiology

Humans are the only known reservoir of poliovirus, which has been found in human faeces, sewage and bodies of water such as rivers, lakes, and streams that have come into contact with raw sewage.

Person-to-person spread of poliovirus via the faecal-oral route is the most important route of transmission, although the oral-oral route may account for some cases. Mainly infected are children under five years of age and children under two compose 75% of the cases [1].

Poliovirus infection typically peaks in the summer months in temperate climates. There is no seasonal pattern in tropical climates.

3- Pathogenesis

Poliomyelitis is a very old disease. Graphic suggestions have even been found in ancient Egypt, although it was only in the middle of the XX century when Weller, Robbins and Enders described the etiologic agent as a transmissible virus able to grow in different cell lines [2]

3.1. Cell adhesion and viral replication

Poliovirus infects human cells by binding to an immunoglobulin-like receptor, CD155, on the cell surface [3, 4]. Initially it adheres to tonsils and gastrointestinal tract cells. The virion is taken up via endocytosis, and the viral RNA is released [5]. The viral positive-stranded RNA can be used as messenger RNA and immediately transferred by the host cell to an internal ribosome entry site (IRES) [6, 7]. Poliovirus mRNA is transferred as one long polypeptide that is then cleaved into mature viral proteins: VP1, VP2, VP3, VP4 proteins of the viral capsid; $3D^{pol}$, an RNA dependent RNA polymerase, whose function is to copy and multiply the viral RNA genome; $2A^{pro}$ and $3C^{pro}/3CD^{pro}$, proteases which cleave the viral polypeptide; VPg (3B), a small protein that binds viral RNA and is necessary for synthesis of viral positive and negative strand RNA; and 2BC, 2B, 2C, 3AB, 3A, 3B proteins, which comprise the protein complex needed for virus replication.

The positive-sense RNA serves as a template for complementary negative-strand synthesis, producing double-stranded replicative form RNA. Many positive-strand RNA copies are produced from the single negative strand. The newly synthesised positive-sense RNA molecules can serve as templates for transference of more viral proteins or can be enclosed in a capsid, which ultimately generates progeny virions. Lysis of the infected cell results in the release of 10,000 to 100,000 infectious progeny virions [8].

3.2. Viral spread

After implantation at a mucosal site and replication in the gut and adjacent lymphoid tissues, polioviruses may disseminate to susceptible reticuloendothelial tissues, via "minor" viremia. In asymptomatic infections, the virus is contained at this point and elicits the formation of type-specific antibodies. In a few infected persons, replication in the reticuloendothelial system gives rise to a "major viremia", which corresponds temporally with a "minor illness" and causes the symptoms associated with abortive poliomyelitis. At this point, the course of poliomyelitis deviates from other enteroviral diseases in the capacity of polioviruses to infect neurons in the grey matter of the brain and spinal cord [1].

The exact routes by which the Central Nervous System (CNS) becomes infected remain uncertain. Three theories, which are not mutually exclusive and require that the virus be present in blood (viremia), have been suggested to explain why. One theory is that the virus passes directly from the blood into the central nervous system by crossing the blood brain barrier, independent of CD155 [9]. A second hypothesis suggests that the virus is transported from the muscle to the spinal cord through nerve pathways by retrograde axonal transport [10, 11]. A third proposition is that the virus is imported into the CNS by infected monocytes or macrophages [8].

4- Clinical features of polio

The following categories classify the extent and seriousness of the disease [1].

Sub-clinical Polio: 90-95 % of cases. The patient is unaware of infection and gains active immunity to further infection from the strain. Sub-clinical polio usually occurs in infants and very young children.

Abortive Polio: 4-8% of cases. It is often characterised by acute respiratory infection or gastroenteritis, but is generally not dangerous. The infection is cut short by the host's defences before it can enter the central nervous system. Symptoms may include: fever, headache, vomiting, diarrhoea, constipation and sore throat.

Non-Paralytic Polio: 1-2% of cases. Symptoms (headache, neck, back, abdominal and extremity pain, fever, vomiting, lethargy and irritability) generally tend to subside after one to two weeks. There are muscle spasms and resistance to flexion in the neck and back and in other muscles throughout the body. The muscles may be tender to palpation.

Paralytic Polio: less than 1% of cases proceed to develop more severe symptoms. In addition to the symptoms of non-paralytic polio, tremors and muscle weakness appear. The flaccid paralysis of muscles innervated by the motor neurons of the spinal cord is the most common type of paralytic polio (Spinal Polio). Usually the paralysis is asymmetrical, and lower limbs are more commonly affected than the upper limbs and trunk. In some cases, the damage involves the neurons in the reticular formation and the nuclei of cranial nerves in the brainstem, which may lead to dysphagia, dysphonia, facial weakness, difficulty in chewing, inability to swallow or expel saliva and respiratory tract secretions (Bulb Polio). Few cases have respiratory muscle involvement, cardiac arrhythmia, instable blood pressure and impaired bladder and bowel function.

5- Global poliomyelitis eradication

In 1988, the 41st World Health Assembly, then consisting of delegates from 166 Member States, adopted a resolution for the worldwide eradication of polio by the year 2000. It marked the launch of the Global Polio Eradication Initiative, spearheaded by the World Health Organization (WHO), Rotary International, the US Centers for Disease Control and Prevention (CDC) and UNICEF [12].

5.1. The strategy

The strategy for global eradication had four elements [13]**.** The first was to strengthen routine immunisation services to optimise population immunity against polioviruses by ensuring that as high a proportion of children as possible received three (subsequently increased to four) doses of oral polio vaccine (OPV) as early as possible in infancy. The second element was to use the annual National Immunisation Days (NIDs) to interrupt the major chains of indigenous poliovirus transmission. NIDs consisted of 2 rounds of supplementary OPV immunisation, 4-6 weeks apart during the low season for enterovirus, targeting all children under five for

additional doses of OPV, regardless of their prior immunisation history. The third element was to report and investigate all cases of acute flaccid paralysis in children under fifteen. Finally, large-scale house-to-house mop-up immunisation campaigns with OPV were organised to interrupt any remaining chains of poliovirus in a country or area.

5.1.1. Vaccines

The OPV vaccine was chosen instead of the inactivated poliovirus vaccine (IPV) for both routine and supplementary immunisation activities [14]. Both vaccines were effective and had been used successfully in the occidental world. Nevertheless, the OPV was cheaper and could be easily administered.

Inactivated Salk Vaccine (IPV). The formalin inactivated intramuscular polio vaccine (IPV) is of high potency and purity. It is both safe and effective. IPV does not induce local IgA mediated immunity to polioviruses in the gut but has been shown to confer herd immunity against poliovirus. It has been used for the last 40 years in some European countries, alone or in combination with OPV. No adverse affects have been reported [15].

Live Attenuated Vaccine, **Sabin Vaccine (OPV).** The live attenuated oral polio vaccine has several advantages over IPV, especially in developing countries: it induces secretory IgA formation and thus, local immunity against reinfection in the pharynx and gut; it is cheaper and needs no expensive sterile equipment nor trained medical personnel for administration; it is a passive transfer of vaccine virus to protect non-immunised individuals [16].

The OPV vaccine is extremely effective, as shown by the dramatic decrease in poliomyelitis since its introduction in Europe and N. America. However, the vaccine strains, in particular, type 3 strains (VP3), can revert to virulence and cause disease in those who have just been vaccinated [17]. Outbreaks have been reported in Hispaniola (2000), The Philippines (2001), Madagascar (2002 and 2005), China (2004), Indonesia (2005) and Egypt (throughout the 1980s) [18, 19]. It is estimated that vaccine-induced poliomyelitis is seen at a rate of 1 in 3,000,000 vaccinations. The actual frequency is greater in immunocompromised children and adult males.

Although the response rate to OPV vaccination is close to 100% in developed countries, it is poor in some developing countries, ranging from 50 - 90% for each strain. It is not entirely clear why this should be so, although a loss of potency of the vaccine due to failure in refrigeration is possible.

5.1.2. Surveillance of all acute flaccid paralysis

The surveillance of wild poliovirus has been carried out through reporting and laboratory testing of all acute flaccid paralysis cases among children under fifteen years of age. Every year, the poliomyelitis surveillance officers collect specimens from over 60,000 paralysed children under the age of 15 in more than 190 countries [20]. Surveillance for acute flaccid paralysis is underpinned by a network of 145 laboratories in 90 countries that isolate and genetically characterise polioviruses, provide an understanding of their geographical distribution, and guide response activities [21]. These laboratories use standard equipment, reagents and methods. They must be reaccredited every 1-2 years to remain in the network and are distributed around the world [22].

5.1.3. The evolution of global polio eradication

Global polio eradication presented, *a priori,* some added difficulties to the previously achieved smallpox eradication. The necessity of several dosages of vaccine to effect immunisation against the virus and the frequently asymptomatic infection made the goal of eradication difficult. In 1988, 125 countries had endemic polio and 350,000 cases were declared.

The immunisation programmes produced excellent results in industrialised countries, where most of the high risk population was vaccinated. Hence, the last detected case in the Americas was in Peru in 1991. In 1999, polio cases had diminished by 99%, with 7,000 cases throughout the world. The European cases had ceased (the last one in Turkey in 1998), as had those in America, the seaside Pacific and most of Africa. Nevertheless, there were cases of wild virus transmission in some counties like India and east Africa**.** These areas had factors that favoured virus survival: poverty, dense population, tropical climate, unsanitary conditions, political insecurity and lack of support by the local community.

It was established that the 3-4 dosages of OPV that confer herd immunity to most people vaccinated in the USA and in "other clean countries" were not enough for these tropical countries, which needed at least 8 dosages per child. However, the transmission still continued when 85-90% children had been immunised, while in some areas of Africa, the poliovirus transmission stopped with a 70-80% immunisation.

Polio cases decreased very slowly and by the end of 2000 only 3,500 cases were reported. WHO changed the deadline of the eradication to 2005. In summer 2000, there was a polio outbreak in Hispaniola. The virus came from an endemic country and the strain derived from that used in the OPV [18]. The outbreak was a

consequence of the decrease of the vaccination on the island by 20-30%, so there were many susceptible children.

The 11th September 2001 bioterrorist attack in New York changed the concept of disease eradication forever. Until then, the *sine qua non* of any eradication effort was to stop vaccinating, reap the financial windfall, and most likely, destroy the virus itself. However, since that moment, it was questioned whether the vaccine program should be stopped once the wild virus was eradicated, leaving the population without protection against a possible bioterrorist attack with poliovirus [23].

A devastating outbreak in India in 2002 discouraged those expecting a near eradication, as in 2001 only 200 cases had been reported in the country.

In April 2003, there were only six endemic countries: Afghanistan, Egypt, India, Niger, Nigeria and Pakistan. Ninety-five percent of the cases were located in India, Nigeria and Pakistan, and within those three countries, transmission was restricted to five major hot spots that accounted for 75% of the global total. It was decided to focus the monetary resources on these countries, so the partners adopted the risky decision to stop mass campaigns in some 100 polio-free countries, enabling them to redeploy forces to the endemic areas. This decision left much of the world vulnerable to a viral reintroduction. At the same time, the partners would continue the campaigns in several polio-free countries considered at high risk for an outbreak because of their proximity to reservoirs, large populations, and low levels of routine OPV coverage: Angola, Bangladesh, the Democratic Republic of the Congo, Ethiopia, Nepal and Sudan.

The vaccination program in Nigeria failed. Less than 50% of the children were vaccinated and the situation worsened when, in July 2003, several Muslim leaders began to protest that the vaccines were tainted with the AIDS virus and sterility drugs, part of a US plot to decimate the Muslim population. The Kano state government cancelled the vaccination rounds planned for September 4-5 [23].

The discontinuation of mass vaccination campaigns in the majority of polio-free countries left these areas vulnerable to importations of wild poliovirus (WPV) from the remaining countries where polio was endemic. Thus, the importation of this virus into at least six of Nigeria's neighbouring countries was reported.

On January 15, 2004 the WHO released an updated Global Polio Eradication Initiative Strategic Plan outlining activities which would: first, interrupt poliovirus transmission globally; second, achieve global certification of polio eradication, and third, prepare for global cessation of childhood vaccination with OPV [24]. To attain the first objective, supplementary immunisation activities (SIAs) in children under five were intensified in endemic countries and in those countries with imported cases. To accomplish the second objective, there was an improvement of the surveillance quality, especially in those countries that had not achieved certification-standard surveillance. Finally, WHO outlined the development of policies for the postcertification era, including detection and notification of circulating poliovirus as public health emergencies, long-term containment of all poliovirus strains, polio vaccine stockpiles and outbreak response mechanisms, as well as routine vaccination.

Progress toward global poliomyelitis eradication was made in 2005. Multiple innovations were implemented during that year, including the relicensing and use of monovalent type 1 (mOPV1) and type 3 (mOPV3) oral polio vaccines, which have greater efficacy against wild poliovirus type 1 (WPV1) and wild poliovirus type 3 (WPV3), respectively, than trivalent OPV [25, 26], and improved quality of SIAs. The number of countries with endemic polio decreased to four (Afghanistan, India, Nigeria and Pakistan). India and Pakistan reported approximately half as many cases in 2005, compared with 2004. However, the total cases reported globally were higher in 2005 than in 2004, because of the three large outbreaks after importation of Nigeria polioviruses into previously polio-free countries. Ninety-four per cent of the cases worldwide were caused by viruses that originated in northern Nigeria [27]. In addition, the largest number of paralytic polio cases known to be caused by circulating vaccine-derived poliovirus occurred on a small island off East Java in Indonesia. Again, a low OPV coverage enabled neurovirulent vaccine-derived poliovirus to emerge and circulate [28].

The global incidence of polio was unchanged from 2005 to 2006, and the number of counties where wild poliovirus transmission had never been interrupted remained the same [27, 29, 30, 31]. The strategies considered for progressing were: a large-scale use of mOPV1vaccine [32], targeted programmes to reach more children through SIAS, and introduction of new laboratory procedures to confirm cases more rapidly [33].

In 2007, more than 400 million children were immunised in 27 countries during 164 SIAs. However, limited progress has been made toward interrupting WPV transmission in Pakistan and Afghanistan. Although the number of children receiving the oral vaccine has increased, the transmission of WPV continues in remote areas affected by ongoing conflict and security problems, where up to 20% of children are not vaccinated [34].

6- Current situation

Since 1988, when the Global Polio Eradication Initiative was launched, the number of cases has fallen by over 99%. In 2008, only 4 countries in the world remain polio-endemic, with persistent pockets of polio transmission: northern India, northern Nigeria and the border between Afghanistan and Pakistan, although the

WPV1 transmission has been curtailed substantially in these regions. The rest of the world is not free from risk because the poliovirus can easily be imported into a polio-free country and can spread rapidly among unimmunised populations. The most recent data published by WHO on poliovirus cases globally are shown in tables 1. Polio surveillance is at historical highs, as represented by the timely detection of cases of acute flaccid paralysis.

Table 1- Global cases. Data as of 13 May 2008 (http://www.polioeradication.org/casecount.asp).

Total cases	Year-to-date 2008	Year-to-date 2007	Total in 2007
Globally	423	155	1312
In endemic countries	402	130	1206
In non-endemic-countries	21	25	106

Presently, it can be said that with global collaboration and sustained commitment, the world may soon achieve global polio eradication.

Acknowledgement This work has been supported by the "SAI de Microbiología"

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Methods – Quantitative Models and Bioinformatics in Microbiology

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A brief note about the effect of microbial growth rate on the assimilation of toluene by *Acinetobacter sp*.

Tatyana Avramova*, Blaga Angelova, Lilyana Boyadjieva, Sava Mutafov

Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Acad. G. Bonchev St., Block 26, 1113 Sofia, Bulgaria

*Corresponding author: e-mail: avramovat@yahoo.com

Studied was the activity of the enzymes involved in the bacterial assimilation of toluene by carbon source limited *Acinetobacter* sp. grown in titrostat under strictly controlled conditions with specific growth rates ranking from 0.015 to 0.109 h^{-1} corresponding to metabolic rates (g toluene assimilated by 1 g cells per hour) from 0.038 to 0.144 h^{-1}. The study confirms the supposed catabolic pathway of toluene by *Acinetobacter* sp. via benzoate, benzaldehyde and catechol. The activities of two enzymes of this pathway were found to increase with the rise of microbial growth rate: benzaldehyde dehydrogenase and catechol-2,3-dioxygenase (C23O). Observed was a sharp increase of the specific C23O-activity with the rise of microbial metabolism from nearly maintenance rate, 0.022 h^{-1}, to 0.046 h^{-1} which could be explained with the generally accepted view that the *meta* pathway catalyzed by C23O provides considerably more energy needed for the activated constructive metabolism of the organism.

Keywords BTEX; VOC; toluene; benzylalcohol dehydrogenase; benzaldehyde dehydrogenase; catechol-1,2-dioxygenase; catechol-2,3-dioxygenase; continuous cultivation; titrostat

1. Introduction

The volatile organic compounds (VOC) from the BTEX-group (benzene, toluene, ethylbenzene and xylene) are pollutants with a high impact on the environment. Biological degradation of BTEX by various microorganisms is the only natural friendly way for their elimination from the environment.

A great number of bacterial strains are known to degrade aerobically toluene and related substrates via several alternative pathways [1-7]. Some denitrifying bacterial strains are described to metabolise toluene via anaerobic pathways [8-9]. Both, aerobic and anaerobic microbial growth on toluene were recently described for a bacterial strain identified as *Thauera* sp. [10].

The aerobic degradation is of particular interest since it is a basic factor for toluene removal from industrial waste gases [11-12].

There are many investigations elucidating the gene regulation of the enzymes involved in the initial steps of toluene degradation and there is an evident lack of studies describing the process in connection with microbial growth kinetics as a knowledge necessary for correct understanding and proper control of all practical applications based on microbial activity. The rare publications bridging in some extent these subjects describe the process from the viewpoint of one only empirically chosen microbial specific growth rate [4]. Possible reason of this fact is the very low water solubility of all BTEX compounds and their manifested growth inhibiting properties which restrain the effective application of the chemostat cultivation technique as the only cultivation method providing effective control over microbial growth rate. To overcome this disadvantage we developed a modification of the chemostat, named *titrostat* [13- 14]. Using this method we selected a rapidly growing toluene utilising bacterial culture and made an attempt to see if there is any correlation between microbial growth rate and the expressed activities of some enzymes of the toluene degrading pathway.

2. Materials and Methods

2.1. Microorganisms

The bacteria used in this study originated from river water. Before the experiment they were never stored on organic media and were never subjected to any genetic manipulations. The experimental culture was obtained after several month continuous cultivation in titrostat on mineral salt medium with toluene as a single source of carbon and energy [13]. The resulted culture was dominated by two *Acinetobacter* species: *A. calcoaceticus* and *A. radioresistens*, persisting in a ratio of *ca* 3:1. The strains are deposited in the Bulgarian Bank for Industrial Microorganisms and Cell Cultures as *A. calcoaceticus* BBIMCC 3645 and *A. radioresistens* BBIMCC 3646.

2.2. Cultivation technique and parameters

The cultivation was carried out in titrostat (**Fig. 1**). In the titrostat the growth-limiting substrate (toluene in this case) enters the cultivation space as a manually adjustable single-compound flow. The microorganisms grow and acidify the culture liquid with a rate corresponding to toluene addition rate. The pH-control module corrects the pH shift by adding titrant solution containing NH_4OH and all non-limiting nutrients.

Complete description of the equipment used, the medium composition and the start-up of cultivation were reported previously [13].

The following parameters were kept constant throughout the study: working volume 2 L, concentration of NH_3 in the titrant solution 1.875 g L^{-1}, air flow rate 120 L h^{-1}, temperature 32 °C, pH 6.8, agitation 1100 min^{-1}. Toluene was added into the culture trough the air port with rates from 0.260 to 1.577 g L^{-1} h^{-1}.

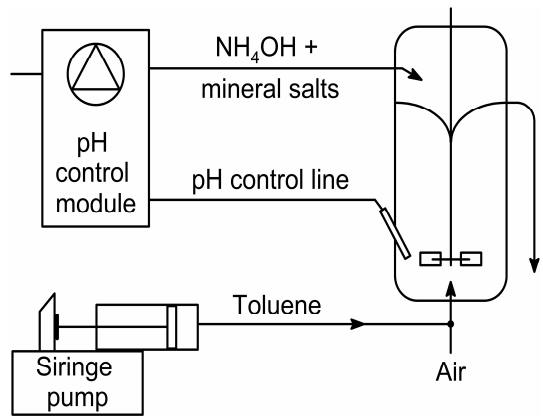

Fig. 1. Principle scheme of the titrostat.

2.3. Analytical procedures

The enzyme activities were estimated for cells taken at steady-state conditions. Criterion for proving the steady state was the invariability of the flow rate and the biomass concentration for at least three changes of the working volume of the titrostat.

The assays of biomass and toluene were described previously [13].

Protein was determined by the method of Lowry [15].

The activities of benzylalcohol dehydrogenase (BADH), benzaldehyde dehydrogenase (BZDH), catechol-1,2-dioxygenase (C12O) and catechol-2,3-dioxygenase (C23O) were determined in cell free extracts. They were prepared at 4 ˚C by sonification of cells suspended in 100 mM phosphate buffer, pH 7.5, containing 10 % (v/v) acetone followed by centrifugation at 16000 g.

The activities of BADH and BZDH were measured according to the methods described by Worsey and Williams [16]. C12O was measured according to the method of Varga and Neujahr [17]. C23O was measured according to the method Nozaki [18]. *p*-Cresol methylhydroxylase and *p*-hydroxybenzyl alcohol dehydrogenase were determined according to the methods described by Hopper and Taylor [19].

2.4. Quantifications

The specific microbial growth rate, μ, was estimated in steady state conditions when it equals the dilution rate, D, (h^{-1}): $D = F/V$, where: F and V are the titrant flow rate (L h^{-1}) and the working volume of the titrostat (L), respectively.

The yield coefficient, Y_s, (gram biomass obtained from one gram toluene) was defined as ratio of the biomass formation rate to the rate of toluene utilisation: $Y_s = (FX)/(F_s - QS_e - FS)$, where: F_s stands for the toluene addition rate (g h^{-1}), Q is the aeration rate (L h^{-1}), S_e is the concentration of the toluene in the exit air (g L^{-1}) and S stands for the residual toluene concentration (g L^{-1}).

The effect of the specific growth rate on the biomass yield was described by the known Pirt equation [20]: $Y_s = (Y_s^m \mu)/(\mu + Y_s^m m_s)$, where m_s is the rate of substrate expenditures for cell maintenance (grams substrate used by one gram biomass in one hour, h^{-1}) and Y_s^m is the maximal theoretical yield (grams cells obtained from one gram assimilated toluene which would take place in the absence of expenditures for cell maintenance). The maintenance coefficient, m_s, was calculated by the linearized form of Pirt equation, $1/Y_s = 1/Y_s^m + m_s/\mu$, using the common least squares technique. The metabolic coefficient, q, (g substrate assimilated by one g cells in one hour, h^{-1}) was estimated as a ratio of the specific growth rate to the corresponding biomass yield: $q = \mu/Y_s$ [21].

For BADH, BZDH and p-HBADH one unit activity, U, was defined as the amount of enzyme catalysing the reduction of one μmol NAD in one minute.

For C12O and C23O one unit activity, U, was defined as the amount of enzyme catalysing the production in 1 minute of 1 μmol cis,cis-muconate or 2-hydroxymuconic semialdehyde, respectively.

The specific activity of all enzymes was expressed as units activity per mg protein.

The numerical data for enzyme activities presented below are mean values of at least three independent measurements.

3. Results and Discussion

3.1. General notes

The experimental results obtained in this work are summarised in **Table 1**. It should added that in our previous experiments it was found out that at the conditions providing biomass concentration 10.8 g L^{-1} a steady state of the cultivation system can be achieved at growth rate up to 0.176 h^{-1} corresponding to toluene addition rate 2.588 g L^{-1} h^{-1}. The maximum biomass yield from assimilated toluene, Y_S^m, was 0.883 and the maintenance coefficient, m_S, was estimated to 0.022 h^{-1}. At higher toluene addition rates the cultivation system was not stable due to limited oxygen transfer capacity of the equipment used [13]. For the purposes of the present study we selected specific growth rates from the range under 0.11 h^{-1}.

There were two reasons for this decision: a) All soil and aquatic microorganisms (including *Acinetobacter* sp.) persist in their natural habitats in conditions of strong limitation for sources of carbon and energy. The most low specific growth rates provide metabolic rates commensurable with the maintenance rate thus bringing the experimental conditions near to the natural ones. b) At metabolic rates, q, close to the maintenance rate, m_S, there is a negligible risk of accumulation of any intra- or extra- cellular intermediates which could compromise the observation.

At the growth rates listed in **Table 1** residual toluene as well as intermediates originating from its degradation were not detected in the cultivation space.

Table 1. Effect of microbial specific growth rate (μ) on biomass yield (Y_S) metabolic coefficient of bacterial growth (q) and specific activities benzylalcohol dehydrogenase (BADH), benzaldehyde dehydrogenase (BZDH), catechol-1,2-dioxygenase (C12O) and catechol-2,3-dioxygenase (C23O)

μ , h^{-1}	Y_S	q , h^{-1}	Specific enzyme activities, U/mg protein			
			BADH	BZDH	C12O	C23O
0.015	0.396	0.038	2.1	11.4	20.5	203
0.019	0.414	0.046	3.1	10.7	26.9	728
0.042	0.634	0.066	1.6	12.5	71.9	727
0.058	0.659	0.088	2.7	50.0	127.6	998
0.070	0.699	0.100	4.5	31.9	27.0	847
0.109	0.755	0.144	2.0	30.2	24.7	664

3.2. The pathway of toluene assimilation

The analysis of the cell free extracts in our experiments revealed relatively high activities of two key enzymes belonging to upper pathway bacterial aerobic assimilation of toluene. These are BADH, responsible for dehydrogenation of benzylalcohol to benzaldehyde, and BZDH catalyzing its further dehydrogenation to benzoate.

Our attempts to detect any of the enzymes belonging to the known alternative pathways such as p-cresol methylhydroxylase and p-hydroxybenzyl alcohol dehydrogenase using cresols and hydroxybenzoates as substrates gave negative results. In our opinion, this is a reliable indication that microbial culture utilises toluene via the pathway passing through benzylalcohol and benzaldehyde as shown on the scheme (**Fig. 2**). The same upper toluene degrading pathway for *A. calcoaceticus* was reported earlier by MacKintosh and Fewson [22] and Chalmers *et al.* [23].

Two more enzyme activities were detected in the cell free extracts, C12O and C23O, indicating that the further degradation of benzoate results in formation of catechol which undergoes a split of aromatic ring. The analytical results show that both C12O and C23O exist simultaneously.

It is notable that the enzymes of the studied degradation pathway have different ranges of specific activities which increase with any new step of degradation process (**Table 1**). The results show also a parallel rise of their sensitivity to the metabolic rate of biomass formation, q.

3.3. The balance between *ortho* and *meta* routes

The presence of two alternative reactions leading to a split of the aromatic structure of catechol is not a feature typical for *Acinetobacter* sp. only. It was reported [24] that *Alcaligenes eutrophus* also assimilates catechol by both intradiol ring fission catalyzed by C12O (*ortho* pathway) and extradiol ring fission catalyzed by C23O (*meta* pathway). In this, at low growth rates the assimilation proceeds via the *ortho* pathway while at high growth rates the metabolism goes through the meta route.

Estimating the growth rate in transient-state conditions of sodium benzoate grown C-limited genetically modified strains *Pseudomonas putida* Kiesel & Müller [25] demonstrated recently that the apparent maximum growth rate of the organisms was 1.3 times higher for strains using the *meta* pathway compared to the *ortho* pathway using ones. The authors adduce solid arguments in favour of the conception that the increased values of the maximum growth rates observed are direct consequence of the fact that the *meta* pathway is able to provide considerably more energy for the constructive metabolism.

Our experimental data (**Table 1**) show that the extradiol split of the catechol aromatic ring always prevails over the intradiol reaction even at the most low specific growth rate tested, 0.015 h^{-1}. At this specific growth rate the value of the metabolic coefficient, $q = 0.038\ h^{-1}$, is close to the maintenance rate, $m_s = 0.022\ h^{-1}$. It could be supposed therefore that the increased demand of energy for microbial growth is the reason of the sharp rise of C23O specific activity. This point of view is in a good accordance with the facts set forth above concerning the specific role of the *meta*-pathway in microbial metabolism.

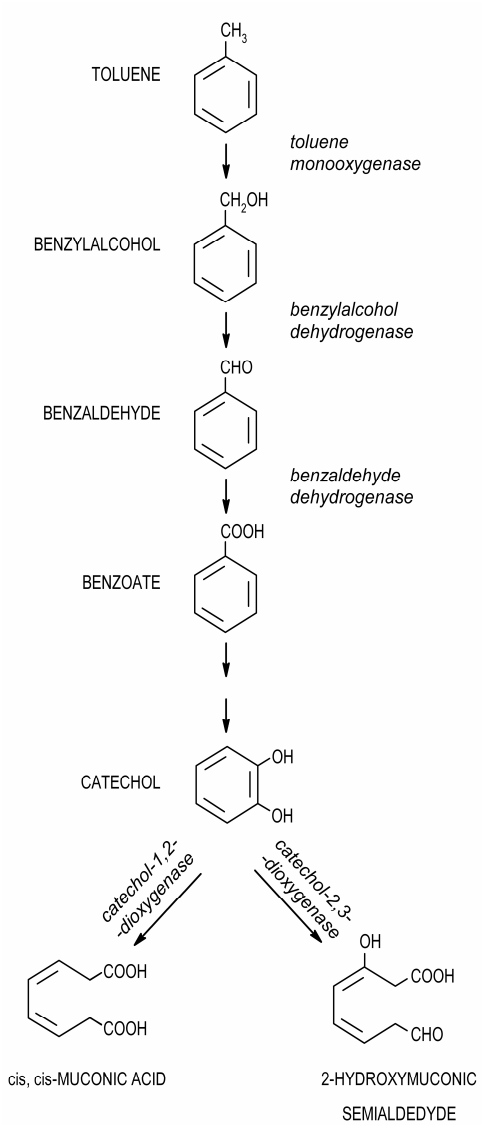

Fig. 2. The pathway supposed for toluene assimilation by *Acinetobacter* sp.

Acknowledgements This work was supported by EU Commission according to the contract IC15 CT96 0710 "Purification of waste gases".

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Application of artificial neural networks to predict ochratoxin A accumulation in carbendazim-treated grape-based cultures of *Aspergillus carbonarius*

F. Mateo[1,*], **A. Medina**[2], **R. Gadea**[1], **E.M. Mateo**[2], **F.M. Valle-Algarra**[3], **R. Mateo**[3], and **M. Jiménez**

[1] Dpto. de Ingeniería Electrónica, Universidad Politécnica de Valencia, Camino de Vera 14, E-46022, Valencia, Spain.
[2] Dpto. de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain.
[3] Dpto. de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain.

[*]Corresponding author: e-mail: fermaji@teleco.upv.es, Phone: +34 678071185

Ochratoxin A (OTA) is a mycotoxin showing serious toxicological (even carcinogenic) properties that occurs in a variety of food products. The capability of artificial neural networks (NNs) to forecast OTA accumulation over time in cultures of *Aspergillus carbonarius* has been explored for the first time in this work. Grape-based cultures of *A. carbonarius* were analysed for OTA by liquid chromatography. The input factors to the NNs were temperature, water activity, concentration of the fungicide carbendazim and time. The outputs were OTA levels in cultures. Multi-layer perceptrons (MLP) and radial-basis function (RBF) NNs were studied. The lowest mean square errors (MSE) for training and test were obtained by MLP trained by Bayesian Regularization without validation. Performance was lower when "hold-out" validation was accomplished. RBF NNs only gave similar results to the best MLPs when the number of nodes was 200. Using the best NNs the predictability of OTA concentration in the cultures was excellent. Logarithmic transformation of raw OTA data was unsuccessful.

Keywords Artificial neural networks; ochratoxin A; predictive analysis; *Aspergillus carbonarius*

1. Introduction

Economic losses arising from plant diseases caused by phytopathogenic fungi are associated with yield reductions. However, fruit, vegetable, and cereal quality and safety may also be adversely affected, undermining both consumer confidence and profitability to the producer. One of the main objectives for producers and researchers is to avoid the contamination of plant-derived foods and animal feed with mycotoxins, which are compounds with toxic properties towards humans and animals, causing acute and chronic effects. Many phytopathogenic fungal species produce mycotoxins [1-4]. Ochratoxin A (OTA) has been reported to be a nephrotoxic and carcinogenic mycotoxin and is produced by various species of *Aspergillus* and *Penicillium* [5, 6] Recently, *A. carbonarius* has been demonstrated to be the mould responsible for contamination of wine, grapes, grape juice and vine fruits with OTA [7]. The maximum level of this mycotoxin in wine has been established at 2.0 μg/Kg [8]. Carbendazim is a systemic fungicide that plays a very important role in plant disease control. It is applied worldwide on crops to control fungi that can lead to plant disease and has been used to control fungal infections produced by various fungi in vineyards.

Artificial neural networks (NNs) are interconnected network structures consisting of many simple processing elements that can perform many parallel computations for data processing [9]. They have been applied to some biological systems and to develop models that could estimate kinetic growth parameters of microorganisms [9-11]. Recently, we have investigated the applicability of these models to predict the levels of deoxynivalenol in wheat [12]. Our aim was to assess the capability of NNs to forecast OTA accumulation over time by *A. carbonarius* on grape juice-based medium at different temperatures, water activities (a_w) and carbendazim concentrations. Application of NNs to predict the accumulation of OTA in food or culture media has not been found in the literature.

2. Material and Methods

2.1 Fungal strain and cultures

The *A. carbonarius* strain used in this study was isolated from wine grapes and is kept in the collection of the Department of Microbiology and Ecology at the University of Valencia. Cultures were performed as described in previous paper [13]. Briefly, stock extract from red wine grapes was modified by mixing 20% extract with 80% of a mixture of water-glycerol of variable composition to provide a_w values of 0.98, 0.96 or 0.94 in the definitive solid media (after addition of agar). After addition of agar, the media were autoclaved, aliquots of a carbendazim emulsion (100 mg/l) were added to obtain the following levels 0 (control), 50, 250, 350 and 450 ng of carbendazim/ml) and media were poured into Petri dishes. A culture of the fungal strain to be inoculated was used to prepare a suspension of $1 \cdot 10^6$ spores/ml. A small volume of this suspension was used to inoculate the media, which were incubated in closed chambers [14]. The incubation temperatures were 20, 25 and 28°C, because optimum temperature for OTA production is about 20°C [15]. Lag phase for growth was considered as the time (days) necessary to grow a colony showing an average diameter equal to 5 mm.

2.2 Ochratoxin A determination

Once the lag phase was reached, the OTA levels in the cultures were determined daily from the 3rd up to the 15th day. The method for determining OTA used liquid chromatography with fluorescence detection [13]. Averaged results of duplicate measurements were used as single output data. Undetectable levels were considered as 0 for computation purposes. All OTA levels were in the range 0 – 6 μg/g.

2.3 NN models

All values in the data set obtained after the determination of OTA in the cultures were used to train and test different NN models, which were evaluated with regard to their performance. Multilayer perceptron (MLP) and radial-basis function (RBF) NNs were assayed. To evaluate the fitting and prediction accuracy the mean-square error (MSE), the root mean-square error (RMSE) function, and the % standard error of prediction (%SEP), were computed by means of the equations 1 to 3:

$$MSE = \frac{\sum (obs - pred)^2}{n} \tag{1}$$

$$RMSE = \sqrt{\frac{\sum (obs - pred)^2}{n}} \tag{2}$$

$$SEP(\%) = 100 \cdot \frac{RMSE}{mean(obs)} \tag{3}$$

where *obs* is observed value; *pred* is predicted value; *mean (obs)* is the mean of observed values and *n* is the number of observed values. Training was optimized according to the MATLAB [16] default criterion, which assumes that the lower the MSE, the better the model mimics the data. Coefficients of determination (R^2) were also calculated. The goal was to minimise the MSE for test (MSE_{test}). The whole data set was split into a training set used to build the NN, a validation set (used to determine the stopping point), and a test set, which is made of samples, not previously shown to the NN, and only used to evaluate the performance of the NN. If validation is omitted, then there are only training and test sets. In that case, if the size of the test set is kept constant the training set can be larger. The more accurately a NN model can predict observed samples that were omitted in the training process, the better the model is.

For all NN assayed raw OTA data and their log transform were alternatively tested for comparison purposes.

2.4 Multilayer layer perceptron NNs

Single-layer perceptrons were built using 4 inputs, 1 layer of hidden neurons and 1 output. They can be noted as 4:N:1, where N is the number of nodes (neurons) in the hidden layer. The input signals were the values for temperature, a_w, carbendazim level, and time. The output was OTA concentration (μg/g) in culture media. N was varied from 2 up to 30. MSE, RMSE, %SEP and the R^2 were obtained and served to analyze the performance of each NN. The program used for model design was the neural network toolbox built in the

MATLAB 7.0 package [16]. The algorithms used were Levenberg-Marquardt (LM), Resilient Propagation (RP) and Bayesian Regularization (BR). For each value of N, various NN models were tested both without and with "hold-out" validation. The data set included 585 samples and was split in different subsets, as explained in sect. 2.3. Without validation, training set included 500 randomly chosen samples, and test set included the remaining 85. With "hold-out", the training set was also composed of 500 samples, the validation set of 40, and the test set of the remaining 45. N determines the complexity of a net: as N increases, computation effort is greater. If N is very high a net can learn the training set perfectly and a zero training error could be achieved (the net over-fits the training data), but this net will usually have a high generalization error on independent test sets.

The architecture of MLP-NNs was $4:N_1:N_2:1$, being N_1 and N_2 the number of nodes in the first and second hidden layers, respectively. The N_2 values were even numbers from 2 to N_1 ($N_2 \leq N_1$), but the sum of hidden nodes was kept to ≤ 32. We began with $N_1 = 10$ and $N_2 = (N_1, N_1\text{-}2, \ldots)$, and continued with $N_1 = 12, 14, 16, \ldots$ and so on. Training was performed with the 3 algorithms and worked without and with validation. The numbers of data in training, validation and test subsets were the same as previously indicated for single-layer perceptrons.

2.5 Radial-basis function (RBF) NNs

This type of NN has only one hidden layer. The number of tested nodes in the hidden layer was varied in steps of 5 up to 80; moreover, a 200-node NN was tested. The data set for training and test included 500 and 85 samples. Twenty NNs were averaged for each architecture. The same statistics as in the case of perceptrons were calculated.

3. Results and Discussion

3.1 Multi-layer perceptron NNs

For MLP NNs the MSE_{test} values were generally lower when the BR algorithm without validation was carried out. The lowest value (0.0029) corresponded to N=26. The LM algorithm without validation gave similar MSE_{test} values and the RP algorithm gave the worse results. Validation usually increased the values of MSE_{test} as compared with the values obtained without validation. Thus, with validation, the lowest MSE_{test} (0.0043) was reached with N=30 by LM algorithm. Table 1 summarizes the values of MSE_{test} and R^2_{test} for some of the best MLP NNs assayed. The lowest MSE_{test} value was obtained by a NN with N_1=18 and N_2 =12 trained by the BR algorithm without validation. Similar values were obtained with N_1=14 and N_2 =12 or N_1=20 and N_2 =10. The BR algorithm also provided a net with the lowest MSE_{test}, when validation was carried out. The LM and RP algorithms provided higher MSE_{test} values.

Table 1 Some of the better MLP-NNs obtained.

Algorithm[a]	Validation[b]	N_1[c]	N_2[c]	MSE_{test}	R^2_{test}
BR	No	18	12	0.0018	0.9982
	No	14	12	0.0019	0.9983
	No	20	10	0.0020	0.9980
	Yes	20	6	0.0028	0.9972
	Yes	18	4	0.0038	0.9969
	Yes	20	8	0.0043	0.9964
LM	No	16	16	0.0093	0.9907
	Yes	20	10	0.0044	0.9955
RP	No	16	14	0.0163	0.9859
	Yes	16	10	0.0296	0.9688

[a] BR: Bayesian Regularization; LM: Levenberg-Marquardt; RP: Resilient Propagation.

[b] No , no validation; Yes, validation was performed by the "hold-out" method.

[c] N1 and N2 are the number of nodes in the first and second hidden layer, respectively

Fig. 1 shows a regression line of predicted OTA levels by the best MLP NN against observed levels. It displays the capacity of the model to accurately predict OTA accumulation in the medium as a function of the input parameters. The data points used to train the model were included and the regression parameters are very close the values corresponding to perfect accurately predictability (slope = 1, intercept = 0). The R^2 value indicates an excellent fit in the studied range.

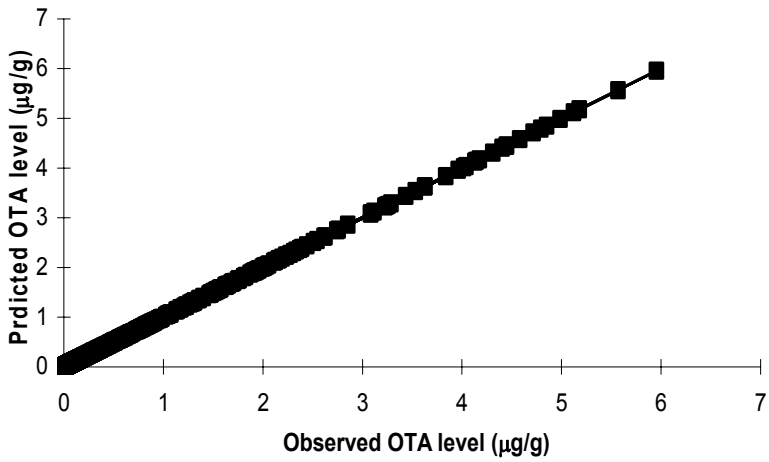

Fig. 1 Predicted OTA levels obtained by the best NN without validation against observed OTA levels for the data set used during training.

For the test set, fit was also showed a good performance. For the best validated NN this procedure was applied to the samples used for training plus validation and test. In this case, the slope and intercept differ a bit more from the theoretical values but the performance is more realistic. Usually validated models show higher errors than the corresponding non-validated models because fewer samples are considered for training, but the first ones are more robust. Log transform of OTA data was not useful because errors were higher than errors attained with raw data.

3.2 RBF NNs

Decrease of MSE with the number of nodes in the hidden layer can be described by a power function. The lowest MSE values was obtained by the 200-node net tested. Except for this net, errors were higher than those found for the best MLP assayed. RBF NNs were less successful than MLP as similar performance needs of a very high number of nodes in the hidden layer. Log transform of OTA data was not useful, as in the case of MLP NNs.

4. Conclusions

NNs are very useful to estimate OTA accumulation in solid grape based media. Among the tested nets the best was a MLP with N_1=14 and N_2=18 trained with the BR algorithm and without validation. If validation is performed, also the BR algorithm gives the lowest errors. Errors are higher when the model is validated. The R^2 value obtained by the best model when plotting predicted against observed OTA levels is high, even for test samples. RBF-NN designs can attain similar performances to MLP, although the number of nodes should be much higher (≥ 200). Log transformation of OTA data always gave place to worse NNs. Because no NN design to predict OTA accumulation in commodities or culture media as a function of the variables here studied has been previously reported, this work is pioneering this kind of research.

Acknowledgements The authors wish to thank financial support from the Spanish Government "Ministerio de Educación y Ciencia" (Project AGL-2004-07549-C05-02/ALI and two research grants) and from the Valencian Government (Conselleria d'Empresa, Universitat i Ciencia) for financing a research grant.

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Evaluation of a Prototype Lateral Flow Device: Serological Test Kit for Rapid Detection of Potato Ring Rot Disease

Nader El-Badry[*,1], Safwat El-Haddad[1] and John Elphinstone[2]

[1] Potato Brown Rot Project, Dokki, Giza, Egypt
[2] Central Science Laboratory, Sand Hutton, York, England

[*]Corresponding author: e-mail: nader.el-badry@alumni2005.ncl.ac.uk, Phone: +20-2-37498673

A prototype lateral flow device (LFD) serological test kit was evaluated at Central Science Laboratory (CSL), York, England for on-site detection of *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*), the causative agent of potato ring rot disease. Monoclonal antibody (MAb) was produced against *Cms* extra-cellular carbohydrate and then was tested for specificity and sensitivity of detection against a comprehensive panel of bacteria composing different isolates of *Cms* and close relatives from the National Collection of Plant Pathogenic Bacteria (NCPPB) and CSL research (Protect System) collection. When the Mab was used in immunofluorescence antibody staining (IFAS) tests, the detection limit was as few as 1×10^3 bacterial cells ml^{-1}. However, in the LFD, the detection limit was recorded as 1×10^4 bacterial cells ml^{-1} in naturally infected and artificially spiked potato, tomato and aubergine plant extracts. All mucoid *Cms* strains were detected using the LFD but the non-mucoid strain of *Cms* (NCPPB 3898) was not detected. Specificity of the LFD was comparable to IFAS and Polymerase Chain Reaction laboratory-based tests in distinguishing between *Cms* and closely-related bacteria.

Keywords Lateral flow device; *Clavibacter michiganensis* subsp. *sepedonicus*; Potato ring rot disease; Monoclonal antibody; Immunofluorescence antibody staining test; Polymerase chain reaction.

1. Introduction

Clavibacter michiganensis subsp. *sepedonicus* is one of the most regulated diseases of potatoes world wide. The organism is often difficult to detect in potato crops because it can be present in symptomless plants and tubers and in low populations and incidence. *Cms* is easily overgrown on agar media by saprophytes. Ring rot, particularly in its latent form, is therefore very difficult to diagnose, especially within the time frames appropriate for taking statutory action [7].

Immunochromatographic assays, also called lateral flow tests, have been around from some time. They are a logical extension of the technology used in latex agglutination tests, which was developed for the first time in 1956 by Singer and Plotz [13].

Serological test kits are usually only used for presumptive diagnoses. Since there are risks of errors due to serological cross-reactions and non-specific background reactions, further laboratory testing using more sophisticated test methods is always required. Nevertheless, an immediate indication of the presence of a particular target organism in a growing or stored crop can assist on-site decision-making and ensure that follow-up confirmatory laboratory testing is efficiently targeted. This will hopefully enable a more efficient front-line defence against the entry and spread of key plant pathogens.

Immunofluorescence Antibody Staining (IFAS) test is a key screening test that involves staining the target bacterial cells with specific antibodies to which fluorescent markers are bounded. Selective binding of the antibodies to the cell walls of the ring rot bacteria allows them to be observed under UV microscopy. IFAS is widely used in Europe for detection of bacterial pathogens in plant materials for the presence of *Cms* and *Ralstonia solanacearum*, the causal agents of ring rot and brown rot in potato plants respectively [7]. It is also used as the principal brown rot screening test in Egypt [8]. In the Netherlands, IFAS is used to screen 60,000 seed potato samples annually. IFAS is also used to detect many other bacterial pathogens, for example in France it is used to screen tomato seed lots against the bacterial canker pathogen, *C. michiganensis* subsp. *michiganensis*.

2. Materials and methods

2.1. Culture and storage of bacteria

All *Cms* and related strains were originally obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, Central Science Laboratory (CSL), York, UK) and stored in glycerol cryoprotectant media at -80ºC using the Protect™ Bacterial Preservation System (Technical Service Consultants Ltd).

A panel of isolates of *Cms* as well as related bacteria and non-related bacteria strains were chosen to represent diverse dates and countries of origin (Table 1).

Cms and related bacteria were raised on yeast glucose medium (YGM-agar) [3]. Cultures were incubated at 18^{o}C for 7 days. Nutrient glucose agar (NGA) medium was used to culture all non-related bacteria and incubated at 28^{o}C for 48 hours. *R. solanacearum* was cultured on triphenyl-tetrazolium-chloride (TTC) agar medium [11] and appeared as white, fluidal colonies with pink centers after 48 hours incubation at 28^{o}C [6].

2.2. Preparation of bacterial suspensions

Cells were suspended in SDW using a sterile loop and adjusted to standard optical density of 0.1 at 650 nm; the approximate viable bacterial population of this suspension was estimated at 10^8 CFU per ml^{-1} and was confirmed by dilution plating on YGM medium. Serial dilutions of cell suspensions were performed in SDW; 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 CFU per ml^{-1}.

2.3. MAbs produced at the Central Science Laboratory (CSL)

Antibodies were harvested from a series of selected cell lines producing MAbs to the ring rot bacterium. These antibodies had been previously prepared using *Cms* isolate NCPPB 4053 to three types of antigens: Glutaraldehyde fixed whole cells, water-soluble extracellular polysaccharide (EPS), and Cell wall extracts.

2.4. Determination of the specificity, sensitivity and titre of the IFAS test using different MAbs

Three newly produced CSL- MAbs and a commercial monoclonal antibody were evaluated in three replicates for three times. Dilution series of the whole bacterial panel (Table 1) were examined in IFAS according to Janse J. [9] to determine the minimum detectable populations and the potential for cross-reactions or for false positive results.

Sensitivity of detection was determined for each MAb using three different *Cms* isolates (NCPPB 4053, 2140 and 3898, CSL, York, UK). Cells were suspended as serial dilutions in sterile buffer saline (PBS) 2.7g $Na_2HPO_4.12H_2O$, 0.4g $NaH_2PO_4.2H_2O$, 8.0g NaCl (0.01 M adjusted to pH 7.2). Dilutions of bacterial suspensions fixed onto multi-spot slides were estimated to contain 10^7, 10^6, 10^5, 10^4 and 10^3 CFU per ml^{-1} according to spectrophotometer readings of $O.D._{650nm}$. All antibodies were used at the optimum working dilution as determined previously. sterile PBS was used as a negative control.

Table 1 The bacterial panel

****PROTECT (Pr) No.**	***NCPPB No.**	**Identification**	**Host or source**	**Accession date**	**Country of origin**
3181	3032	*Clavibacter michiganensis* subsp. *insidiosus*	*Medicago sativa*	Feb-98	Not specified
3244	1064	*Clavibacter michiganensis* subsp. *michiganensis*	*Lycopersicon esculentum*	Feb-98	Italy
3204	2580	*Clavibacter michiganensis* subsp. *nebraskensis*	*Zea mays*	Feb-98	USA
2435	4030	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum tuberosum* (cv. Truffe de Chine)	1997	France
---	3898	*Clavibacter michiganensis* subsp. *Sepedonicus*	*Solanum tuberosum*	Not specified	Ukraine

4700	4292	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum tuberosum*	Nov-02	USA
4595	4053	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum Melongena*	Jun-02	United Kingdom
3322	---	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum tuberosum*	Not specified	USA
5516	---	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum tuberosum*	4-Jan	United Kingdom
3513	3664	*Clavibacter michiganensis* subsp. *tesselarius*	*Triticum aestivum*	1990	USA
3213	255	*Clavibacter tritici*	*Triticum aestivum*	Feb-98	Egypt
4426	4110	*Curtobacterium albidum*	*Soil, Chinese paddy*	Oct-01	China
1074	844	*Curtobacterium flacumfaciens* pv. *Poinsettiae*	*Euphorbia pulcherrima*	1960	USA
2455	---	*Erwinia chrysanthemi*	*Solanum tuberosum* (cv. Carlingford)	Sep-97	United Kingdom
3985	---	*Erwinia caratovora* subsp. *atroseptica*	*Solanum tuberosum*	Jan-01	United Kingdom
3981	---	*Erwinia carotovora* subsp. *carotovora*	*Solanum tuberosum*	Jan-01	United Kingdom
3445	---	*Erwinia carotovora* subsp. *carotovora*	*Solanum tuberosum* (cv. Atica)	Dec-98	Not specified
5106	---	*Clavibacter* sp.	*Ribes* sp	Jul-03	United Kingdom
4140	---	*Ralstonia solanacearum*	Soil	Feb-01	Kenya

***NCPPB No.** National Collection of Plant Pathogenic Bacteria (NCPPB, CSL, York, UK).

**** PROTECT No.** Protect™ Bacterial Preservation System (Technical Service Consultants Ltd).

2.5. Validation of the prototype LFD test kit for *Cms*

Sensitivity and specificity of detection of *Cms* was evaluated using the prototype kit using the bacterial panel. Suspensions containing approximately 10^6 CFU per ml^{-1} of the whole panel except *Cms* and used for specificity testing and suspended in the blocking buffer, named Buffer C (CSL Pocket Diagnostics, York, UK). 65 µl of suspension was added to the wells of each LFD test. However, tenfold serial dilution of *Cms* isolates in buffer C were used for sensitivity testing. Artificially (spiked with *Cms* isolate No. Pr 4595) and naturally (infected with *Cms* isolate No. Pr 3322) treated plant tissue extracts suspended in blocking buffer (Buffer C, CSL Pocket Diagnostics, York, UK). Buffer C contains phosphate buffered saline, Tween 20 detergent, 0.05% sodium azide as a preservative and polyvinylpyrrolidone (PVP). Finally, to simulate on-site field testing, stem and petiole sections from *Cms*-infected and healthy potato plants and aubergine seedlings (CSL, York, UK) were shaken for 2 minutes in 5 ml buffer C in extraction bottles provided with the LFD kit and three drops of the resulting suspension was added to wells of each LFD test (Fig. 4).

For interpretation of the results (Fig. 3), there were two blue lines (control line (C) & test line (T)) appeared within 3 - 5 minutes when the test was successfully performed indicating a positive result. The appearance of only one blue line (C) within 5 minutes indicated a negative result.

2.6. Target DNA purification from plant extracts

DNA was purified from plant tissue extracts containing *Cms* using a commercial purification kit (Promega Wizard® Magnetic DNA Purification System for Food, USA) according to the manufacturer's instructions.

2.7. Quantitative real-time (TaqMan®) polymerase chain reaction [TaqMan-PCR] for detection of *Cms*

PCR was performed in a 96-well format Using 7700 ABI Prism sequence detector (Applied Biosystems, USA). Standard TaqMan-PCR reaction conditions were used [12].

The TaqMan test procedure was performed with *Cms*-specific DNA primers and fluorogenic probe according to Schaad *at al*. [12]. From each diluted bacterial suspension 90 µl was added to 10 µl of 500 mM NaOH to give a final NaOH concentration of 50 mM. After heat treatment to 96 ^{o}C on a heated block for 15 min, samples were placed on ice for 20 min and then diluted tenfold to 1 ml using ultra sterile distilled water (USDW) to dilute out the NaOH.

The reaction mix contained (per 25 µl reaction) 2.5 µl of 10X PCR Buffer (Amplitaq-Gold, Applied Biosystems), 3.5 µl 25 mM $MgCl_2$, 2 µl of 200 µM dNTP mix (containing dATP, dTTP, dCTP, dGTP), 0.75 µl each of 10 µM forward primer CMS50-2F and 10µM reverse primer CMS133R [12], 0.125µl *Taq* polymerase (5U per µl Amplitaq Gold) and 12.80 µl of USDW using an electronic pipette. To each reaction, 2 µl of sample (crude cell lysate) were added.

As for sensitivity of detection, the number of cells present in *Cms* dilution series was quantified by real-time TaqMan-PCR assay of Schaad *et al*. [12]. The results of six *Cms* isolates are shown in Fig. 2. The limit of reliable detection was 10^3 cells per ml (<10 cells per PCR reaction volume) with a maximum Ct of 40 cycles representing no detection.

2.8. Production of infected plant materials

2.8.1. Inoculation of potato plants with *Cms*

Three different cultivars of potato tubers [vrs. Hermes, Pentland Dell and Desiree] were inoculated with *Cms* isolate no. Pr 3322.

Sprouted potato tubers were immersed in the bacterial suspension or were stabbed with needles coated with bacterial colonies for 15 min. Also, seedlings inoculated with sterile PBS and were used as negative controls. Inoculated tubers were then planted in 30-cm diameter pots containing compost and incubated in quarantine growth room conditions at 18-24ºC for up to 28 days. During this period, plants were observed for the development of typical wilting symptoms and isolation of the bacterium from symptomatic and asymptomatic plants was attempted.

2.8.2. Spiking of potato, tomato and aubergine extracts with *Cms*

Cms isolate Pr 4595 was suspended in SDW and the viable bacterial population was adjusted to 10^8 CFU per ml^{-1} spectrophotometrically according to the $O.D._{650nm}$. Tissue samples (Including 1 cm stem and petiole sections and 1 cm^2 leaf pieces) were removed from healthy potato plants, tomato (var. Moneymaker) and aubergine (var. Black Beauty). One ml bacterial suspension was added to the samples in sterilised small plastic bags (~10x10 cm) which were then crushed using a Homex grinder (Bioreba Ltd., Switzerland) and the supernatant was then used for testing. Negative control extracts were prepared in SDW. All extracts were tested using IFAS, TaqMan PCR and LFD tests and results were compared for positive and negative results, possible cross-reactions (false positive results) or inhibition (false negative results).

3. Results and discussions

3.1. Characterisation and validation of new MAbs for use in IFAS test

The three new MAbs reacted very similarly in all tests performed, indicating that they may have affinity to the same bacterial epitope.

Sensitivity of detection of *Cms* in IFAS test using all MAbs was equivalent to those obtained using polyclonal antibodies by Janse and Van Vaerenbergh [10]. Reliable detection of a minimum population of 10^4 cells per ml of *Cms* was achieved.

No cross-reactions were recorded with bacteria closely related to *Cms* during MAb specificity evaluation and when repeated three times. However, false positive results were recorded with one strain of *C. michiganensis* subsp. *tesselarius* (*Cmt*) only at high concentrations of MAbs above the determined optimum working dilution. *Cmt* is a plant pathogenic bacterium which causes bacterial mosaic of wheat and is present only in the USA [1]. At the optimum working dilution of MAbs, cross-reactions were also observed with isolates of *C. michiganensis* subsp. *insidiosus* (*Cmi*) and *C. michiganensis* subsp. *nebraskensis* (*Cmn*). *Cmi* and *Cmn* are also plant pathogens

[1] causing bacterial wilt of alfalfa and leaf blight of various monocotyledonous hosts (sugar cane, sudan grass, sorghum and maize respectively). *Cmn* is restricted to North America but *Cmi* has been found across Europe and worldwide.

The commercial antibody did not cross react with *Cmi* or *Cmn* and was therefore found to have higher specificity to *Cms*, which was proved after repetitions three times. As a result, the newly produced antiserum is helpful for screening large numbers of samples however confirmation of positive findings can be more efficient by using the commercial antiserum.

3.2. Validation of prototype LFD tests

The LFD was assembled at CSL incorporating the newly selected MAb that was produced against the bacterial EPS (Strain no. NCPPB 4053) on both the latex particles and the target line (Fig. 1). Furthermore, no cross-reactions were observed when testing bacterial suspensions of the non-related potato pathogen *R. solanacearum*. Clear control lines were produced when the relevant anti-mouse antibody was used but not when mixtures of anti-mouse and anti-rabbit antibodies were used in a previous study. It was also evidence during the preliminary work that the use of buffer C is required to prevent non-specific binding to the *Cms* antibodies observed when non-*Cms* bacterial suspensions or healthy plant extracts were tested in PBS only.

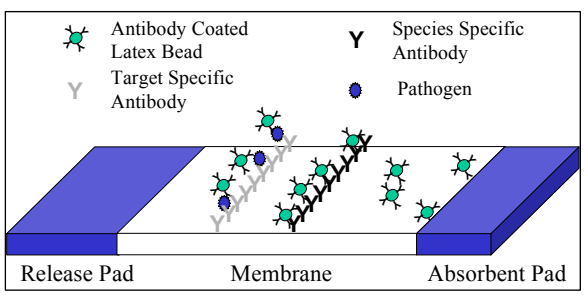

Fig. 1 The mechanics of the lateral flow device (Danks & Barker, 2000)

This study has evaluated the use of new MAbs to *Cms* in the LFD test kit format previously described by Danks and Barker [2] for detection of viral and other diseases. Validation data obtained show that a prototype LFD assay enables detection of *Cms* with sensitivity and specificity of detection similar to that obtained using the standard laboratory IFAS method. However, the antibody used in the kit did not have affinity for non-mucoid strains of *Cms* (e.g. NCPPB 3898). These results are consistent with those obtained by De Boer [4] in which a similar MAb produced against purified *Cms* EPS De Boer *et al*. [5]. Such strains have been found to cause natural infections of potato ring rot disease and it is therefore important also to detect during screening tests.

3.3. Sensitivity of detection of *Cms* using a prototype LFD test

Sensitivity of detection of five of the seven *Cms* isolates, in the LFD test was equivalent to that observed when tested by IFAS, with the limit of reliable detection at 10^4 cells per ml^{-1} and unreliable detection at 10^3 cells per ml or lower. However, two *Cms* isolates were not detected at any concentration (NCPPB 3898 and NCPPB 2113). The optimum concentration for detection was around 10^6 cells per ml. Clear control lines were found in all tests performed.

3.4. Sensitivity and specificity of detection using quantitative real-time PCR (TaqMan-PCR)

The results obtained for the sensitivity and specificity of *Cms* strains were equivalent to those obtained by the original authors [12]. Sensitivity of reliable detection was comparable to that obtained in IFAS and LFD tests and was limited by the low sample volumes (2 µl) used in each PCR reaction. All *Cms* isolates tested were detected with the exception of the isolate NCPPB 2113. This isolate was also not detected in the LFD test and was later found not to be *Cms* but a contaminant bacterium which had out-competed the *Cms* in culture. Complete lack of cross-reactions with closely-related or non-related bacteria confirmed the usefulness of this highly-specific real-time PCR assay for laboratory confirmation of *Cms* identity.

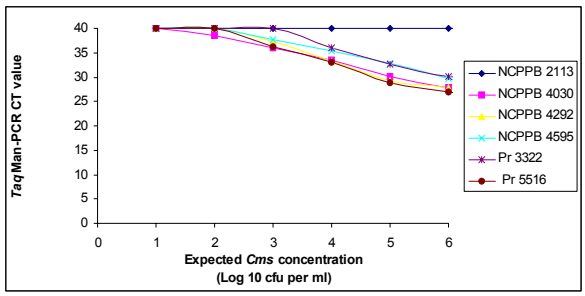

Fig. 2 Ct values (PCR cycles at which amplification is first detected) obtained when testing dilution series of 6 *Cms* isolates by real-time TaqMan-PCR.

3.5. Comparison of detection methods

The results obtained demonstrated that it was possible to detect *Cms* in plant extracts using all three detection assays (IFAS, prototype LFD and real-time PCR). No cross-reactions were observed with any of the closely related bacteria to *Cms* or with the other bacterial pathogens of potato when tested at 10^6 cells per ml^{-1}. All the spiked leaves of potato and tomato varieties by *Cms* (isolate No. Pr 4595) gave clear positive results after it was tested by IFAS using all MAbs. Also, the leaves of the spiked aubergine by the same *Cms* isolate gave a positive result although it was not as clear as the previous tow spiked plants. Prototype LFD test gave a clear positive result when the spiked potato (Herm variety) tested by the same *Cms* isolate in addition, the control line (C) for this kit was obviously clear. TaqMan-PCR confirmed all positive results obtained from IFAS or LFD tests. Each method had advantages and disadvantages. The IFAS assay was potentially prone to false positive results with other subspecies of *C. michiganensis* or, perhaps more significantly, with related soil-borne bacteria which are more likely to be present in potato samples. However, no false negative results were seen with the IFAS test when the asymptomatic potato plant (variety Pentland Dell) diagnosed, which is an essential requirement for a screening test. But the same was not recorded when the commercial MAb was used. Whereas, false-positive results can be later identified in more extensive confirmatory testing. Neither LFD nor real-time PCR assays gave false positive results with healthy plant extracts but false negative results were recorded in each case. With the LFD assay, it was apparent from sensitivity testing that the isolate NCPPB 4030 gave the clearest results at optimum concentrations (10^6 cells/ml) but these results were less clear as concentrations increased or decreased from the optimum. False negative results were obtained with two of the three potato cultivars, regardless of the type of tested plant parts. These results are difficult to explain without further study but it is possible that certain cultivars (e.g. Desiree and Hermes) contain high levels of PCR inhibitors in their tissues.

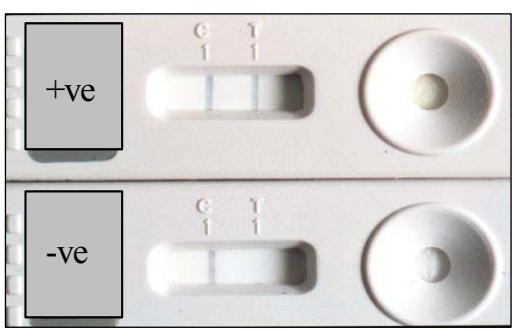

Fig. 3 Development of the LFD test result (Danks & Barker, 2000)

An actual positive and negative result using a Pocket Diagnostic device (results obtained after 3 minutes after sample addition)

+ve (2 blue lines - control & test (C&T)) indicates a positive result

-ve (1 blue line - control only (C)) indicates a negative result

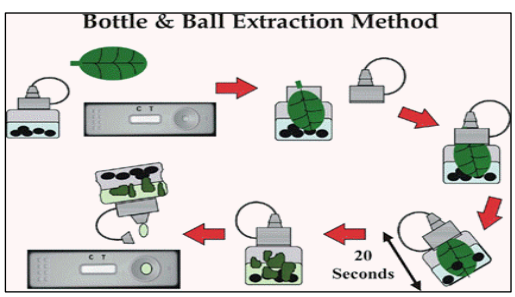

Fig. 4 The sample extraction method takes 30 seconds (Danks & Barker, 2000)

Whole or parts from plant leaves are placed into the bottle containing extraction buffer. The bottle is shaken for 20 seconds, then a few drops are added to the LFD kit.

4. Conclusion

A combination of methods is recommended when detecting *Cms* in plant samples. The LFD test is more appropriate for on-site preliminary diagnosis, whereas the IFAS is preferred as a first laboratory screening test and the specificity of the TaqMan-PCR test is valuable for laboratory confirmation of positive screening tests.

Acknowledgements This research was carried out at Central Science Laboratory (CSL), York, England through an EU-Egyptian project, Potato Brown Rot Project Phase II (SEM 03/220/51A ; EGY 1B/1999/0192; CRIS ref 1999/055-089) , Dokki, Giza, Egypt and with collaboration with scientists at CSL; D. Stead, C. Danks, G. Johnson, V. Tomkies, J. Flint, S. Malbin and I Barker. The author would like to express special thanks to all his family, especially his parents for their sincere encouragement.

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Expression, purification and characterization of the precursor of human pulmonary surfactant protein B (preproSPB) produced in *Escherichia coli*

Elisa J. Cabré. F Gil-Dones, Jesús Pérez-Gil

Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain

Pulmonary surfactant protein B (SP-B) is involved in the transfer of phospholipid molecules from specific lipid/protein assemblies produced by pneumocytes to form surface active films at the air-liquid interface of lungs. The lack of SP-B is lethal, being its absence associated with an irreversible respiratory failure at birth. In vivo, SP-B is synthesized as a larger precursor, preproSP-B, of 381 amino acids, which suffers proteolytic processing to remove N-terminal and C-terminal flanking propeptides during the exocytic pathway ending in the secretory lamellar bodies as the mature active 79-residue (9 kDa) polypeptide. Human preproSP-B has been cloned and expressed in strain BL21 (DE3) of *E.coli* and the recombinant His-tagged preproSP-B form of 42 kDa has been purified by affinity chromatography. After electrophoretic analysis and Western Blot detection, a preliminar structural characterization has been carried out by different spectroscopic methods with the ultimate objective of analyzing how the protein behaves under physiologically relevant environmental conditions. Production at high yield of preproSP-B, followed by a limited proteolysis will offer new possibilities in the development of the next generation of entirely synthetic therapeutic surfactants.

Keywords pulmonary surfactant, surfactant protein B, propeptide, saposin-like domain

1. Introduction

Pulmonary surfactant is a lipid/protein complex synthesized and secreted by the type II pneumocytes of the alveolar epithelium of lungs. This complex spreads as a thin film at the air-liquid interface, reducing the surface tension of the water layer covering the respiratory surface and avoiding alveolar collapse at the end of expiration. Absence of an effective surfactant is associated with severe respiratory pathologies such as the neonatal respiratory distress in premature infants or the acute respiratory distress syndrome in infants and adults suffering of acute lung injury (*1*). It is well known that the phospholipid fraction of pulmonary surfactant contains the most surface active components. However, presence of specific surfactant-associated proteins, and particularly of surfactant protein B (SP-B), is strictly required to ensure a very rapid adsorption of phospholipids into air-liquid interface (*2;3*).

SP-B is an amphipathic protein of 79 residues and 8-kDa, essential for pulmonary function (*4*). Membrane binding, destabilization and fusion promoted by SP-B (*5;6*) make this protein important in modulating surfactant structure and function *in vivo*. These membrane-active properties of SP-B, as well as its homology with saposins (sphingolipid activator proteins), point to a potential role of the protein as a lipid transferring protein from surfactant storages into the interfacial monolayer over the aqueous film covering the respiratory tract (*7;8*).

SP-B is matured from at least three proteolytic steps (*9*) (*10*) of the 381-amino-acid (42-kDa) precursor proSP-B. The two propeptides flanking the mature protein module at its N- and COOH-terminal sides are cleaved to release the active form, which is the one assembled into lamellar bodies, the organelles where pulmonary surfactant is stored until secretion. The specific function of these two flanking sequences is still not well established, although it has been proposed that these two hydrophilic domains could shield the hydrophobic membrane-active SP-B fold from a potential deleterious effect against endogenous cell membranes. The N-terminal propeptide has been shown as necessary and sufficient for proper intracellular trafficking of proSP-B and assembly and secretion of mature SP-B in surfactant membranes, (*11;12*) suggesting that this flanking propeptide could play a role as a chaperon-like element until the last step, leading to the insertion of mature SP-B in surfactant lipid membranes, coupled with biogenesis of lamellar bodies. Expression in a null SP-B background of a proSP-B construction containing the N-terminal but not the C-terminal propeptide restored lung function and surfactant composition indicating that the C-terminal domain is not essential for SP-B processing and mice survival (*13*). The possibility that the C-terminal cleaved domain could play further roles as an independent protein in the alveolar spaces cannot be discarded.

It is known that the full SP-B precursor contains three saposin-like domains, which presumably fold independently, including one in the mature sequence and two at the N-terminal and the C-terminal propeptides,

respectively. Presence of an additional saposin A-type domain can be also detected in the N-terminal domain. The N-terminal propeptide of human proSP-B has been already produced and purified from a heterologous system (*14*). The final purpose behind the heterologous expression of different human proSP-B forms is the development of strategies to produce massive amounts of recombinant active human SP-B, potentially useful to design new entirely synthetic therapeutic surfactants. Production of recombinant proSP-B variants will also improve possibilities to investigate the molecular processes associated with pulmonary surfactant assembly.

The goal of the present work is to obtain enough quantities of purified human proSP-B to approach a detailed characterization of its structure and lipid-interacting properties.

2. Materials and methods

2.1 Expression and purification of rpreproSP-B

Strain DH5αF´ of *Escherichia coli* was used for plasmid amplification and BL21 (DE3) cells were used as the protein expression host. Cells were maintained in LB medium containing 1% (w/v) bactotryptone (Sharlau), 0.5% (w/v) yeast extract (Scharlau) and 1% (w/v) NaCl supplemented with 100 μg/mL ampicillin (Ap) (Ecogen). Solid LB contained 1.5% agar (Scharlau). When needed, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Applichem) was included in the medium at 50 μg/mL as well as 4 μL of 800 mM isopropyl-β-D-thiogalactopyranoside (IPTG, from Genaxis).

cDNA sequence coding for human preproSP-B (Genbank® Accession No. M24461) was cloned into the framework *Nde*I-*BamH*I of the bacterial expression vector pET15b (this construct was kindly provided by Dr. J. Johansson from Uppsala University, Sweden) (Fig. 1).

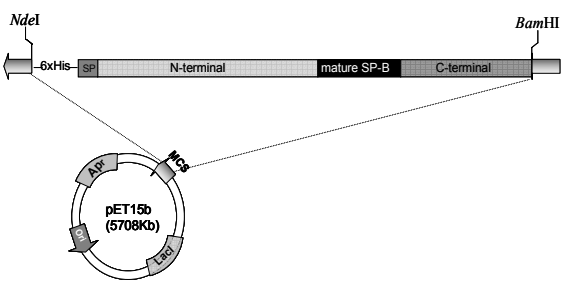

Figure 1.-Structure of pET15b cloning vector containing the human sequence of SP-B precursor. The construct contains the sequence encoding human pulmonary surfactant protein B preproprotein (GenBank® Accession No. M24461) cloned between the NdeI–BamHI restriction sites of pET15b vector. This pET15b vector includes a reiterative cytosine, adenosine, thymidine sequence at the 5′ end of the multicloning cleavage site (MCS) resulting in the incorporation of 6 His at the NH2-terminus of the recombinant protein. The lactose promoter and the lacI gene preceding the ribosome binding site (RBS) allow high level of expression of the recombinant protein under a IPTG-promoted tight regulation.

Plasmid DNA purified by alkaline lysis using Spin Clean miniprep kits (Ecogen) was checked for size in agarose gel electrophoresis with Tris-Acetate-EDTA buffer. Two hundred μL of DH5αF´ competent cells prepared according to a modified method (*15*) were transformed with 1 μL of the plasmid product by the heat-shock procedure , including the addition of 1 mL SOB (2 % ,w/v bactotryptone, 0.5 %, w/v, yeast extract, 10 mM Na Cl, 2.5 mM KCl) and 10 μL of 2 M glucose. After 1 h incubation at 37 ºC, 50 μL of cells were platted on LB containing Ap, X-Gal and IPTG and incubated O/N at 37 ºC. Plasmids from putative recombinants were purified and cloned cDNA was checked by sequence analysis. Recombinant plasmids were used to transform competent BL21 (DE 3) cells and the sequence was again assessed.

Protein expression an purification was achieved by adapting the method described elsewhere (*16*). A single colony of *E.coli* BL21 transformed with pET15b/preproSP-B was grown overnight at 37ºC in 25 ml of LB medium containing 50μg/ml ampicilin. A 15 ml aliquot of the culture was inoculated in 500ml of LB medium containing 50μg/ml ampicilin and was grown to an OD_{600nm} of around 0.7. Induction was then performed by addition of 0.5mM IPTG final concentration. Cell culture was then further incubated for 3-4 hours at 37ºC and harvested by centrifugation at 5000 rpm for 15 min. The bacterial pellet was suspended in 10ml of buffer A (5mM Tris-HCl, 150mM NaCL, 12% w/v sacarose, 1% PMSF, 10 mM β-mercaptoethanol, pH 7.3). Once resuspended, the cells were sonicated in a water bath ultrasonifier and cycling reeze-thawed 5 times. After centrifugation at 15000 rpm for 15 min, the supernatant was saved and the pellet was resuspended in 15 ml of buffer A containing 1% SDS. The samples were finally dialysed in Spectra/Por ® (MWCO: 3.5) membranes against the Niquel column (HisTrap Ni, 5ml, Amersham) equilibration buffer (NaH_2PO_4 20mM, 500mM NaCl, 5mM Imidazol pH 7.3) before applying them onto the affinity chromatography columne of an Äkta ® purifier (Amersham) device. After loading the lysate, the column was washed with five volumes of equilibration buffer,

then washed with the same buffer supplemented with 50mM imidazol and finally elution of attached protein was carried out with 250mM imidazol in the same buffer.

2.2 Electrophoresis and Immunodetection

SDS-PAGE was performed as described (*17*). Proteins were either dyed with Coomassie or electroblotted (*Western blotting*) to Hyperfilm ECL membranes (Amersham Pharmacia Biotech). Membranes were blocked O/N with 0.1 % Tween 20 and 3 % (w/v) milk in PBS and incubated with monoclonal His-tag peroxidase conjugate (SIGMA) at a final dilution 1/8000 for 90 min at room temperature. Alternatively, membranes were incubated with an anti-proSP-B_N primary monoclonal antibody (a gift of Prof. Timothy Weaver, University of Cincinnati, USA) diluted 1:2000, and then with a secondary anti-Mouse IgG peroxidase-conjugate antibody (SIGMA) diluted 1:1000 to detect an N-terminal epitope of recombinant proSP-B. Also, commercial peroxidase-conjugated anti-His-tag monoclonal antibodies were used . Blots were washed with PBS containing 0.1 % Tween 20, and proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.3 Protein analysis

Total protein concentration was quantitated by the method of Lowry (*18*), using bovine serum albumin (Sigma) as standard. Amino acid analysis of hydrolyzed *r*preproSP-B samples was performed on a Beckman 6300 automatic analyzer to accurately determine protein concentration.

2.4 Circular dichroism

Far-UV circular dichroism (CD) spectra were obtained in a Jasco-715 spectropolarimeter equipped with a xenon lamp. The protein concentration was 0.25 mg/mL and the optical path length 0.1 cm. The CD spectra of the protein were obtained in Tris-HCl, Mes, Sodium Acetate 5mM NaCl 150mM pH 7. The contribution of the buffer was always subtracted. A minimum of four spectra were accumulated for each sample. Values of mean residue ellipticity were calculated on the basis of 110 as the mean molecular weight per residue and are reported in terms of [θ] (degrees×cm^2×$dmol^{-1}$). The secondary structure of the protein was evaluated by computer fit of the dichroism spectrum into four simple components (α-helix, β-sheet, turns and random coil) using the CDPro software package containing three commonly used programs: SELCON3, CONTIN/LL and CDSSTR (*19;20*).

2.5 Fluorescence spectroscopy

Fluorescence studies were performed on a SLM Aminco AB-2 spectrofluorimeter. The protein concentration was 0.1 mg/mL. The buffer used was Tris-HCl, Mes, Sodium Acetate 5mM pH 7, containing NaCl 150mM. The contribution of the buffer was always subtracted. Excitation was performed at a wavelength of 275 or 295 nm, and emission spectra measured over a range of 300–440 nm. The excitation and the emission slits were set at 2 nm and 4nm respectively. The tyrosine contribution to the emission spectra was calculated by substracting from the emission spectra measured by excitation at 275 nm, the emission spectra obtained by excitation at 295 nm multiplied by a correction factor. This correction factor was obtained from the ratio between the fluorescence intensities measured with excitation at 275nm and 295nm at emission wavelengths higher than 380 nm, where there is no tyrosine contribution.

2.6 Mass spectrometry analysis

MALDI-TOF MS analyses of protein samples were performed in a Voyager-DETM STR instrument (PerSeptives Biosystems) fitted with a 337 nm nitrogen laser and operated in reflector mode with an accelerating voltage of 20000 V. All mass spectra were calibrated externally using a standard peptide mixture (Sigma).

3 Results

3.1 Expression and purification of rprepro-SP-B

We have cloned, expressed and purified the human precursor of pulmonary surfactant protein SP-B in BL21 (DE3) cells. After three hours of induction the cells were collect by centrifugation and lysed by different denaturant strength buffers, as it has been described in Matherials and Methods. Samples from the different steps after lysis and solubilization were analyzed by 12% acrylamide gel electrophoresis. After cell lysis with

5mM Tris-HCl buffer pH 7.3, containing 150mM NaCl, 12% w/v sacarose, 1% PMSF, and 10 mM β-mercaptoethanol, low quantities of protein were detected (figure 2 lanes 4). Inclusion of 1% of SDS in the lysis buffer produced higher yields in protein solubilisation (figure 2 lane 3).

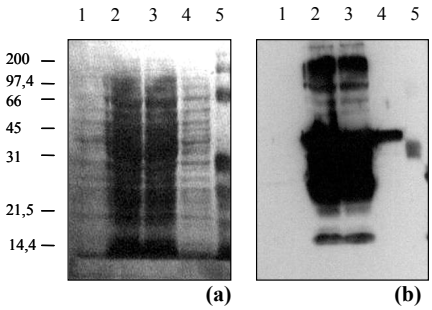

Figure 2.-12% SDS-PAGE gels (a) Coomassie Blue stained and (b) analysed by Western Blot and inmunostaining with anti-Histag antibodies. Lane 1: Bacterial cells before induction, where no expression is detected. Lane 2: cells after 3 hours post-induction, where the expression of rpreproSP-B with the expected molecular weight mobility, was detected. Oligomeric forms with higher apparent molecular weights and shorter protein forms due to incomplete translation were also detected. Lane 3: Supernatant after lysis and centrifugation of induced cells, including 1% of SDS in the lysis buffer. Lane4: Supernatant after lysis and centrifugation of induced bacteria cells, when the lysis buffer contains no SDS. In this case only the monomeric form of rpreproSP-B, with 42 KDa, is detected. Line 5: Molecular weight markers.

The bacterial lysate was dyalized to remove the excess of detergent and then loaded onto a Niquel His-trap column in a FPLC chromatography system. The purified protein was analyzed by 12% acrylamide gel electrophoresis followed either by Coomassie blue staining or Western blot analysis (figure 3). SDS-PAGE of purified rpreproSP-B detected a main band after Coomassie blue staining, corresponding to the electrophoretic mobility of the rpreproSP-B monomer. This band was properly recognized by antibodies against both the His-tag extension and human SP-B (figure 3).

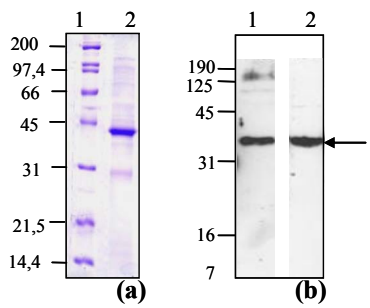

Figure 3.-SDS-PAGE and Western blot analysis of purified rpreproSP-B. (a) 12% acrylamide gel stained with Coomassie blue. Lane 1: molecular markers; lane 2: purified rpreproSP-B. (b) Western blot analysis of rpreproSP-B transferred onto a nitrocellulose membrane from a 12% acrylamide gel and probed with anti-proSP-B (lane 1) or with commercial anti-Histag (lane2) antibodies. The arrow indicates the position of rpreproSP-B monomer (42 kDa). The molecular mass of standard markers is indicated on the left of each panel.

3.2 Characterization of purified recombinant rpreproSP-B

Circular dichroism and intrinsic fluorescence emission spectra of purified rpreproSP-B is shown in Figure 4. The far-UV CD spectrum (Figure 4a) shows 2 minima in ellipticity at ~210 and ~222 nm, indicative of a secondary structure with a significant α-helical contribution. The estimation of the proportion of different secondary structure elements according to the Selcon3, CDsstr and Contin/ll programs resulted in the averaged values presented in the table of figure 4. The protein contains slightly more than 20% α-helix and around 25% beta sheets.

The fluorescence emission spectrum of preproSP-B provides mainly information of the microenvironment of the eigth Tryptophan residues in the protein. This emission spectra (fig 4, b) shows a maximum at around 342 nm,.indicating that at least part of the tryptophans are shielded from the exposure to the solvent. The spectrum is dominated by the contribution of trytophans, suggesting that the protein has a proper fold leading to quenching of the fluorescence from tyrosines.

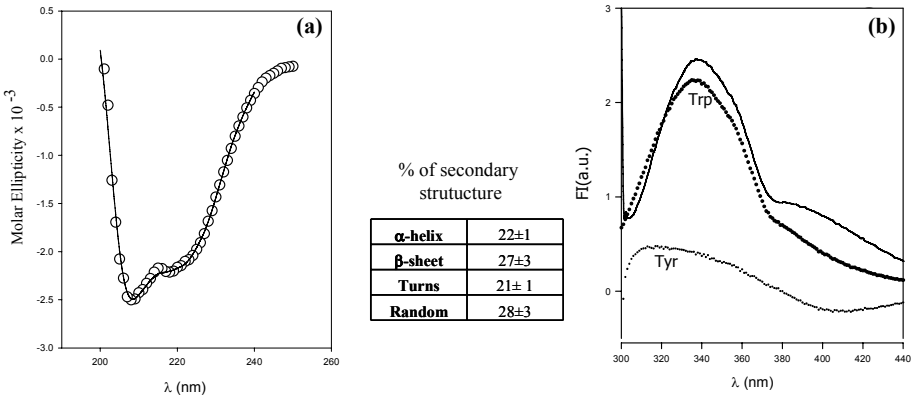

Figure 4.-Spectroscopic characterization of rpreproSP-B. (a) Far-UV circular dichroism spectrum of rpreproSP-B in Tris-HCl 5mM, Mes 5mM, Sodium Acetate 5mM buffer pH 7, containing NaCl 150mM. The protein concentration was 0.3 mg/mL. Symbols are the experimental data and the solid line represents the theoretical spectrum calculated using the CONTIN program (19). The data in the Table summarize the percents of secondary structure of rpreproSP-B calculated from its far-UV CD spectrum using three different programs (Selcon 3, CDsstr and Contin/ll). Results are expressed as means±SD. (b) Fluorescence emission spectrum of rpreproSP-B. The protein concentration was 10 µg/mL in the same buffer and the fluorescence intensity (FI) is presented in arbitrary units (au). Dotted spectra represent the deconvoluted contributions of tryptophans and tyrosines to the global spectrum (solid line), calculated as described in materials and methods.

MALDI-TOF MS analysis of the protein in solution is shown in figure 5, where the monomer and a fraction of dimmers of rpreproSP-B can be detected.

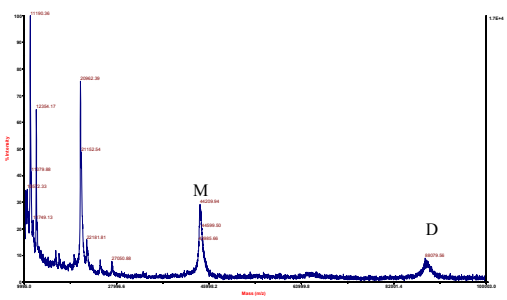

Figure 5- MALDI-TOF MS spectrogram of rpreproSP-B in Tris-HCl 5mM, Mes 5mM, Sodium Acetate 5mM buffer pH 7, containing NaCl 150mM .The peaks corresponding to the monomeric (M) and the dimeric forms (D) are indicated.

4. Discussion

The precursor of human pulmonary surfactant protein SP-B bearing an N-terminal His-tag extension has been expressed in *E.coli* and purified to a large extent by a single metal affinity chromatographic step. The calculated yielding was in the range of 1mg rpreproSP-B per litre of cell culture. The goal of the present study was to use this expressed and purified protein to initiate the characterization of the structure and the behaviour of SP-B precursor under physiologically relevant conditions. Further studies of lipid-protein interactions will be approached to understand the conformational changes suffered by the protein as it is assembled into surfactant membranes under conditions mimicking those in the exocytic pathway.

The circular dichroism spectra show that the purified protein contains about 21% of α-helix, a proportion compatible with the idea that rpreproSP-B is composed of three saposin-like modules (*21*). Preliminar data, including the analysis of the protein by mass spectrometry, suggest that preproSP-B has some tendency to aggregate, forming at least dimers in solution. A partially oligomerized structure could be responsible for the relatively important proportion of β structure in the conformation of the protein. A detailed analysis of the conditions promoting aggregation, as well as the extent of protein oligomerization in different environments, will help to evaluate the importance of protein-protein interactions in the assembly of proSP-B. We have also determined that most cysteines in purified preproSP-B are still in the form of free sulfhidryles. Current work in progress is optimizing formation of the proper disulphides in purified preproSP-B.

The fluorescence and CD spectra of the protein suggest that rpreproSP-B is properly folded, as a form which is soluble but partly oligomerized. Further studies will approach the analysis of the effect of pH, the ionic

environment and the presence of phospholipid membranes on the conformation of the protein. The goal will be to get further insight the mechanisms of assembly of SP-B in surfactant membranes. A parallel line of research will try to optimize production of the mature SP-B module by partial proteolysis of the proSP-B form purified in the present work, as a basis for the design of new therapeutic surfactant preparations.

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Microbial diversity, comparative analysis, and the use of molecular methods in natural environments

J. M. Gonzalez[1], and M.C. Portillo[1]

[1] INSTITUTO DE RECURSOS NATURALES Y AGROBIOLOGIA, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

Recent molecular microbial surveys carried out in natural environments are demonstrating the existence of a huge microbial diversity which is practically undeterminable. Molecular techniques allow the detection of microorganisms without a need for culturing but miss a need for understanding the metabolism of these cells. A number of strategies are available to facilitate our understanding of the microbial community structure and the individual members of that community. Fingerprinting methods are highly valuable for characterizing communities and to compare spatial and temporal trends. This study describes some of the current strategies for the treatment of sequencing data from ribosomal RNA gene libraries and comparison methods to distinguish microbial communities.

Keywords microbial diversity; comparative analysis; diversity index; microbial communities

1. Introduction

While early last Century, the development of bacterial cultures was the focus of attention, the end of last Century faced up a need for culture-independent methods, i.e., molecular techniques based on nucleic acids, to approach the survey of non-cultured microorganisms in natural communities [1]. During the last years, one of the most critical aspects of environmental microbiology is the fact that microbial diversity is too high to be experimentally determine [2, 3].

At present, it is generally accepted that the culture of microorganisms allow for the detection of up to 1% of the total microbial types within a given natural environment. The rest of bacteria might only be approached by using molecular, or equivalent, culture-independent methods. The use of DNA and RNA for the detection of microorganisms is greatly enhancing our understanding of microbial community structure in nature. Howwever, one of the major gaps in environmental microbiology is to build the link between phylogeny and physiology (herein named the PP challenge). This is a consequence of our poor understanding of the great diversity of microorganisms existent in our planet. In fact, most microorganisms on Earth are still unknown and the presence in the environment of some of them has been detected only through their ribosomal RNA gene sequences. They have never been cultured and their physiology and metabolic capabilities are unknown. Their role in the environment, of course, remains to be discovered.

From the proposal of the three major domains of life (Bacteria, Archaea and Eukarya) there has been hope about solving the PP challenge in a near future. Rappé and Giovannoni [4] analyzing the known bacterial diversity pointed out that, at least, half the discovered bacterial phyla have no cultured representatives and many of the rest have only one or few cultured strains. Thus, if we do not advance in our knowledge of the huge variability within the microbial communities in our planet, we will not be able to solve the PP challenge.

In this piece of work, we propose a couple of strategies for the analysis of microbial populations with the aim to obtain satisfactory results from what we know are immensely diverse communities.

2. Our knowledge and DNA databases

Most microbial community surveys are based on the amplification of ribosomal RNA gene fragments and the construction of a clone library from those DNA fragments. Sequencing on selected clones can be performed using several procedures (e.g., reference [5]). Most current surveys are based on the analysis of a relatively low number of sequences [6] although recent strategies allow for the analysis of communities at the thousand level of clones. The results are equivalent using any of these two strategies because of the huge microbial diversity.

Table 1 Advantages and disadvantages of using the whole GenBank database and the alternative database created for this study containing only cultured, well-described species data. The aim is to easily estimate the taxonomic affiliation and closest known metabolic capabilities by homologue searches.

	Whole Genbank database		Alternative database (cultured bacteria)	
Database parameters	Advantage	Disadvantage	Advantage	Disadvantage
Setup required	No			Yes
Majority of "uncultured bacteria"		Yes	No	
Homologue search output	Closest homologue			Closest cultured species
Number of sequences	Maximum available			Only described species
Interpretation by unexperienced users		Difficult	Facilitated	
% Similarity	Highest			Conserved
Analyzed range	All known diversity		Limited to described species	
Length of sequences		Variable	Selectable	
Errors in database		Frequently reported	None/Minimum	
Update	Automatic			Required periodically
NCBI Server needed		Yes	No	

Since a great number of microorganisms (let´s say most of them) are unknown our databases are also lacking that information. A large fraction of 16S rRNA gene sequences in the databases lacks most information on the phylogeny or taxonomic affiliation of the corresponding microorganism. This fact creates a problem when a researcher is looking for the closest homologue [7] to an environmental rRNA gene sequence.

We propose the use of a database only with cultured microbial data. Culture microorganisms correspond generally to well studied and described species. That is, its metabolism is basically known and so the linkage between metabolic capabilities and phylogeny results possible and easy to be estimated. Apparently this would solve the PP challenge, but it all depends on the level of similarity between the query sequence and its closest cultured homologue. We loop back again, since many microorganisms remain unknown a cultured database could also fail to provide with that information. However, a fraction of the problem can be solved at least for a fraction of the microorganisms encountered in a system.

A database constructed for Bast [7] with only cultured microorganisms is available online at http://www.irnase.csic.es/~micro/software.

3. Looking into one's needs

A critical point to think about is to decide what we really need to do in our research. Those of us interested in the comparison of samples over time, or over spatial scales, or even of the effect of specific treatments, could point towards the use of community simplification methods such as fingerprinting techniques. There many of them available. One of the most used methods is the Denaturing Gradient Gel Electrophoresis (DGGE) [8], others are the Temperature Gradient Gel Electrophoresis (TGGE) [9], Single Strand Conformational Polymorphisms (SSCP) [10], terminal Restriction Fragment Legth Polymorphisms (t-RFLP) [11], and several others, some of which have been recently proposed.

While fingerprinting methods will never provide us with a complete vision of a microbial community, they permit to characterize a microbial community through the likely most abundant members of a given community. These fingerprinting methods have drawbacks, but they are an adequate solution to the current puzzle of understanding microbial community dynamics, treatment effects, spatial variations, and some other comparisons among communities. These are needs where the use of community fingerprinting techniques are handy.

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       Length=511
                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

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dbj|AB179509.1|  Uncultured bacterium gene for 16S rRNA, parti...   870    0.0
emb|AJ617855.1|  Uncultured bacterium 16S rRNA gene, clone D14305   862    0.0
emb|AJ292582.1|UEU292582  uncultured eubacterium WD254 partial 16   846    0.0
gb|DQ830567.1|  Uncultured bacterium clone PAS2_E05 16S riboso...   844    0.0
gb|AF523988.1|  Uncultured bacterium clone FW120 16S ribosomal...   841    0.0
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gb|EF075576.1|  Uncultured Firmicutes bacterium clone GASP-WDO...   831    0.0
gb|EF074073.1|  Uncultured Firmicutes bacterium clone GASP-WB2...   831    0.0
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B. Database of cultured microorganisms
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                                                                    Score    E
Sequences producing significant alignments:                         (bits) Value

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AJ233937. Stigmatella aurantiaca 16S rRNA gene, strain Sg a28        250   1e-66
AJ233934. Stigmatella erecta 16S rRNA gene, strain Pd e19            250   1e-66
AJ970180. Stigmatella erecta 16S rRNA gene, type strain DSM 16858T   250   1e-66
AJ233910. Angiococcus disciformis 16S rRNA gene, strain An d4        242   2e-64
AJ233906. Cystobacter violaceus 16S rRNA gene, strain Cb vi34        242   2e-64
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Fig. 1 Example of simplified blast results using as query sequence AJ421903 against the whole GenBank (nr) database (A) and a custom database including only cultured bacteria (B). Sequence AJ421904 corresponds to an Acidobacteria.

Of course, the problems originated to the amplification reactions (generally the PCR or Polymerase Chain Reaction) [12, 13] are also affecting these fingerprinting methods. We must recognize that most methods used today for molecular surveys of natural environments require amplification reactions, so environmental microbiologists are tight with the available methodologies. Other problems associated to fingerprinting methods where the DNA corresponding to specific microorganisms must be separated, generally, by differential migration, could be that often two different microorganisms migrate similarly. As well, eletrophoresis does not always reaches the levels of resolution desired for an ideal discrimination of microorganisms, above all, when working with complex microbial communities.

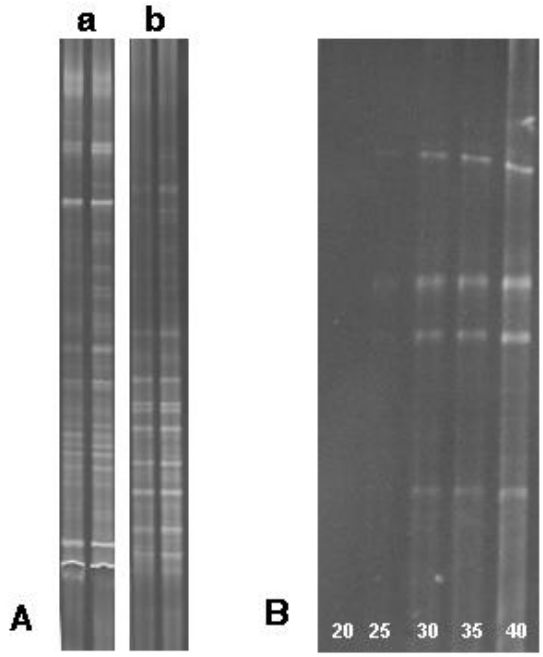

Fig. 2 Bacterial community fingerprints by PCR-DGGE comparing independent replicates from the same environmental sample (A) showing identical profiles for each of the two samples shown in the figure (a, b). A relative quantitative evaluation of the method by PCR-DGGE using an environmental sample (B). Note the exponential amplification at increasing amplification cycles.

Every method has some cons and pros. Current results in our laboratory have demonstrated that the same sample analyzed repeatedly (performing independent replicates) leads to identical (or minor experimental variations) banding patterns (Fig. 2). Thus, a microbial community or sample can be characterized by its own, and unique, fingerprint. The banding pattern of a sample will be exclusive for it and different of the corresponding to any other community. Thus, differences among communities can be easily evaluated if the most abundant members of these communities are being analyzed. Consequently, fingerprinting techniques can be used for the comparison of communities and the visualization of the major representatives of a complex communities, even if we will know nothing about the "rare" fraction, which is the most abundant, of the microbial community.

An advancement in the development of suitable methodologies for the comparison of samples and treatments is the fact that most fingerprinting techniques can be performed following relative quantitative protocols. Several authors have demonstrated the feasibility of this option [14, 15] (Fig. 2). If quantitative results can be obtained, the method will allow to perform comparisons focusing on both the presence/absence of bands (i.e., microorganisms or phylotypes) as well as the intensity of the banding patterns. Band intensity represents the abundance of the corresponding microbial type. Thus, comparisons can be performed based on two factors the presence of a bacteria (or phylotype) and its relative abundance. This is all that is actually required for community comparisons.

When comparing samples, a method to determine if two communities are different or not is required. We have developed a statistical method able to discriminate significantly different microbial communities based on their banding patterns from fingerprinting techniques, providing the level of significance and confirming if the observed differences are due to chance or to biological meaningful effects. The procedure is based on a Cramér-von Mises statistic as described by Portillo and Gonzalez [16]. A computer program, fingshuf, carrying out these estimations can be downloaded from http://www.irnase.csic.es/~micro/software.

4. Future perspectives

Determining microbial diversity is an objective for the next few decades. Knowing what is on our planet is not enough and realizing what each of those microorganisms do must complement standard microbial community evaluations. Once our knowledge of microbial diversity is known, a second objective will be to examine if that diversity corresponds to an established microbial fraction of a community or if it corresponds to allochthonous microorganisms that could be considered and immigrants or individuals just arrived in search of a suitable living environment. Techniques based on evaluating the major components of the microbial communities are likely our best chance to be able to analyze comparatively microbial communities and their dynamism.

If microbial diversity and the metabolic basics of all its members were mostly known, microbial sequence (and their linked metabolic information) databases will be acceptably complete and most of the PP challenge will be resolved. In this scenario, only some of the detected microorganisms from any given environment will

face the challenge of knowing their phylogenetic relationship and their metabolic capabilities. This will be the opposite to the current situation since today most microorganisms are unknown or little is known about them.

Acknowledgements The authors acknowledge financing from the Junta de Andalucía to group CVI288.

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Microbial Physiology, Metabolism and Gene Expression

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Analysis of stability by elements at the proximal 3´-UTR of two *KlCYC1* mRNAs

S. Seoane-Rosende, T. Salgado, M. Poncelas, A.M. Rodríguez-Torres and M.A. Freire-Picos, M.A.*

Area of Biochemistry, Department of Cellular and Molecular Biology. University of A Coruña. Facultad de Ciencias, Campus da Zapateira S/N, 15071 A Coruña. Spain.

*Corresponding author: e-mail: mafreire@udc.es, Phone: +34 98116700, Fax: +34981167065

An important aspect to consider in the modulation of gene expression with biotechnological purposes is mRNA stability**.** The *KlCYC1* gene has a long (1.2 kb) 3´-UTR region that can be used to modulate gene expression in yeast by the alternative use of its proximal or distal 3´-Untranslated Region [1, 2]. The stability of the two *KlCYC1* transcripts was analysed in *Saccharomyces cerevisiae puf3* and *rpb1-1* mutants. When the *puf3* mutant and the deletion of the UGUR element at positions (131-135) were combined, there was a two-fold increase in total *KlCYC1* levels mainly due to the increase in the long transcript signal. After a cease of transcription (*rpb1-1* mutant), the long transcript was stable for more than two hours while the short one for less than one. When the gene was expressed in the yeast *Kluyveromyces lactis* under hypoxic conditions, both transcripts were degraded faster than in the *rpb1-1* mutant. These findings suggest the presence of different mRNA turnover mechanisms able to operate on *KlCYC1* transcripts under different physiological conditions.

Keywords RNA stability; yeast; heterologous expression.

1. Introduction

Apart from transcriptional regulation, mRNA turnover is one of the most important aspects in the regulation of gene expression and it is driven through sequences and secondary structures which are recognised by specific factors [3]. The modulation of gene expression using the 3´-UTR (3´-Untranslated Regions) has been developed thanks to growing knowledge about the regulatory elements included in their sequences [4].

Some genes are transcribed in at least two mRNAs caused by alternative 3´-end processing (cleavage and polyadenylation). Alternative transcripts for a given gene with different 3´-UTR lengths is a feature found in all eukaryotes and that is usually associated with differences in growth state, or is tissue specific, suggesting a regulatory role for these regions [5, 6]. The *KlCYC1* gene has a peculiarly long (1.2 kb) 3´-UTR region that can be used as a model to study alternative RNA-processing mechanisms in the yeast *Saccharomyces cerevisiae* [1, 2]. It is possible to modulate gene expression in yeast by the alternative use of proximal or distal regions of *KlCYC1* 3´-UTR [1].

The stability of *KlCYC1* transcripts had not been tested previously and this aspect needed to be addressed to improve the use of its 3´-UTR elements for heterologous gene expression. As part of the study on the regulatory elements present in this region, we have characterised the sequences determining mRNA stability for both transcripts in two yeast species, *Saccharomyces cerevisiae* and *Kluyveromyces lactis.* For this analysis, we combined site-directed mutagenesis of *cis* elements with the expression in mutants affecting either RNA stability (the *puf3* mutant), the transcription mediated by using an RNA polymerase-II thermosensitive (*ts*) mutant (the *rpb1-1* mutant), or the expression under hypoxia.

2. Materials and Methods

2.1 Yeast strains, media and growth conditions

The *S. cerevisiae* strain used was RS420 (MAT α leu2-3, 112 ura3-52 trp1 his3,4 rpb1-1) [7]. The source of puf3 mutant was the strain CE.ZI22-7A (MAT a ura3-52 his3 Δ1 leu2-3,112 trp1-289 YLL013c::URA3) from EUROSCARF. ZW13 (MATα trp1-1 leu2-3,112 his4-519 cyp3-1 gal$^-$ cyc1$^-$ cyc7$^-$) was obtained from the R. Zitomer laboratory. NRRL-Y1140 from ATCC 8585 was the K. lactis wild type strain used for the genes analysed.

As standard yeast media, cells were grown in YPD (1% Yeast extract, 2% Bacto-peptone, 2% Dextrose). Synthetic Complete Media (CM) was prepared with auxotrophy for either tryptophan or uracil (CM-Trp or CM-Ura, respectively) according to Sherman [8].

The rpb1-1 thermosensitive mutant strain (RS420) is commonly used in stability analyses [9, 10]. This mutant was transformed with plasmid pCAF10, expressing KlCYC1 from a centromeric plasmid [11]. Transformed cells were grown at 30°C and shifted to 37°C for two hours. Samples were taken every 30 minutes.

The hypoxia conditions were generated in a Biostat® MD fermenter (B. Braun Biotech) by bubbling N_2 (99.95% pure, Carburos Metálicos, Spain) using a pO_2 GasMix controller. This allowed us to take samples at different time-intervals without changing the hypoxia conditions.

2.2 Site-directed mutagenesis

Specific mutations on *KlCYC1* 3´-UTR were introduced using the specific oligos and using the Stratagene QuickChange™ site-directed mutagenesis kit.

The plasmid pSS36 containing a deletion in the UGUR element at position 131-134 was obtained using oligos oSSMUT4 and oSSMUT5. Plasmid pCT2 [11] was used as *KlCYC1* template for the site-directed mutagenesis experiments.

2.3 Northern experiments

Total RNA extractions and Northern experiments were carried out according to Zitomer and Hall [12]. The hybridisation signals were quantitated using the Image-Quant program (Molecular Dynamics) and normalised with respect to U3 RNA or ribosomal RNA. The U3 probe, corresponding to *S. cerevisiae* small nuclear RNA (snRNA) R17A, was obtained by PCR amplification with primers U3F: 5´-CGACGTACTTCAGTATGTAA-3´ and U3R: 5´-ATTTGTACCCACCCATAGAG-3'. *ACT1* was amplified with specific oligos. As *KlHEM1* probe, the 0.7 *Pst*I-*Eco*RI fragment from plasmid pMD3.5 was used [13].

3. Results and Discussion

3.1 Elements at the *KlCYC1* 3´-UTR

RSA Tools was used to search for specific consensuses along the *KlCYC1* 3´-UTR (http://rsat.ulb.ac.be/rsat/) [14]. Computer searches of the *KlCYC1* 3´-UTR showed several elements as possible candidates needed to mediate its/mRNA stability (Fig. 1a). Depending on what information was found, the stability of *KlCYC1* transcripts was tested in the following aspects: i) the functionality of the UGUR element (positions 131-135 of the 3´-UTR) (Fig. 1b) and its possible interaction with the Puf3 protein, ii) the role of the AU-rich element (positions 672 to 691 of the 3´-UTR) (Fig. 1c) as possible RNA degradation element, iii) and finally the stability of both transcripts in a physiological change from aerobiosis to hypoxia (this last condition only in *K. lactis*). Both the AU-rich and the UGUR elements are located inside the proximal 3´-UTR (Fig. 1A).

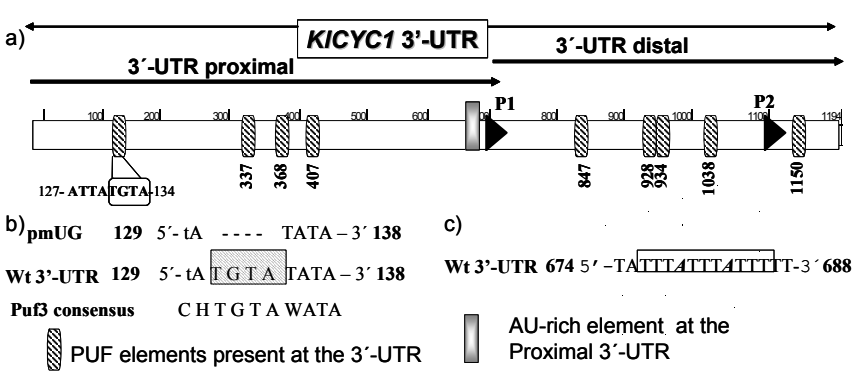

Fig. 1 a) Position of the putative *cis* elements at the *KlCYC1* 3´-UTR related to RNA stability. P1 and P2 are the alternative polyadenylation points. b) Sequences mutated at the proximal 3´-UTR element to analyse its role in Puf3-mediated RNA stability. c) Sequence of the AU-rich element next to the first polyadenylation point.

3.2 Functionality of the UGUR element and its role in a *puf3* mutant background

The Puf protein family (Puf1-Puf5) of *S. cerevisiae* binds to the tetranucleotide UGUR (R=pyrimidine) present in mRNA 3'-UTR regions, potentiating their degradation. Among the Puf factors, Puf3 is involved in the degradation of genes involved in mitochondrial functions [15]. The role of this UGUR element at position 131-135 of *KlCYC1* 3´-UTR and surrounding sequences (AUUNUGUA) matches perfectly with the optimum for Puf3 regulation [16]. Transcript stability was analysed by expressing the gene either with a wild type 3'-UTR (plasmid pCT2) or a deleted form of the UGUR element (mUG) located at position 131-134 (plasmid pSS36) obtained by site-directed mutagenesis with the deletion shown in Fig. 1B. The results are shown in Fig. 2A. When the *puf3* mutant and the deletion of the UGUR element at positions (131-135) were combined, there was a two-fold increase in total *KlCYC1* mRNA levels mainly due to the increase in the long transcript signal (compare lanes 1 and 4).

3.3 Role of the AU-rich element in the proximal *KlCYC1* 3'-UTR

The AU-Rich Element (ARE) is conserved among eukaryotes including the yeast *Saccharomyces cerevisiae* [17]. This element, when located at the 3´-end, is a determinant of mRNA degradation. The *KlCYC1* gene has an AU-rich element next to the polyadenylation site for the proximal transcript (Fig. 1A). The role of this AU -rich element in the stability of *KlCYC1* transcripts was analysed by expressing the gene either with a wild type 3'-UTR or a mutated form of the AU-rich element (pM3 with a deletion) (Fig. 1C). To cease transcription, a thermosensitive *RPB1* mutant (*rpb1-1*) was used. The long *KlCYC1* transcript is clearly more stable than the short one, after two hours there is a clear signal, that disappears in the small one after one hour (Fig. 2B lanes 1 to 4). The mutation of the AU-rich element (data not shown) does not increase the stability of the short transcript, moreover, it destabilises the long transcript containing the distal UTR. Thus, the AU-rich element does not play a degradation role in the 3´-UTR.

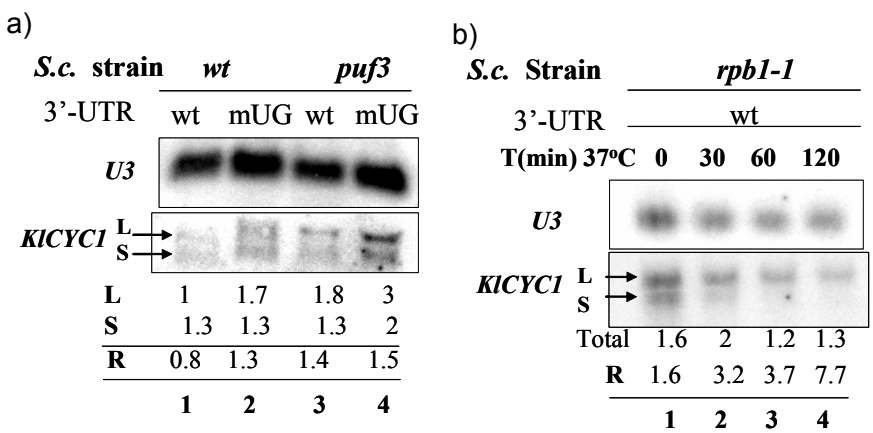

Fig. 2 Analysis of the stability of *KlCYC1* transcripts in *S. cerevisiae*: a) Involvement of the *KlCYC1* UGUR element (UTR position 131-134) and its functional relationship with the Puf3 factor. The 3´-UTR mUG indicates cells expressing a deletion of the UGUR element, b) stability of *KlCYC1* transcripts in the *S. cerevisiae rpb1-1* mutant. Wt: wild type 3´-UTR. Total: quantitated mRNA signals of the two *KlCYC1* transcripts. R: Ratio between quantitated individual signals of long (L) and short (S) *KlCYC1* transcripts.

3.4 Stability under hypoxia for *KlCYC1*, a cytochrome *c* encoding gene

We also considered expressing *KlCYC1* in its natural background, the aerobic respiratory yeast *Kluyveromyces lactis*. When hypoxia is induced, there is no transcriptional activity in this gene [18].

After inducing hypoxia, as described in Materials and Methods, samples were taken at the periods indicated in Fig. 3. RNA was extracted and analysed by Northern blot. We included in our analysis as *KlHEM1* another aerobic gene involved in heme biosynthesis [19] and as *KlACT1* one that is functionally unrelated (Fig. 3). The two respiratory-related aerobic genes have a similar response under hypoxia. The ratio small/long *KlCYC1* transcripts shows a faster degradation of the small transcript, especially when comparing lanes 3 and 4 (Fig. 3). After one hour, there is no *KlCYC1* transcript signal.

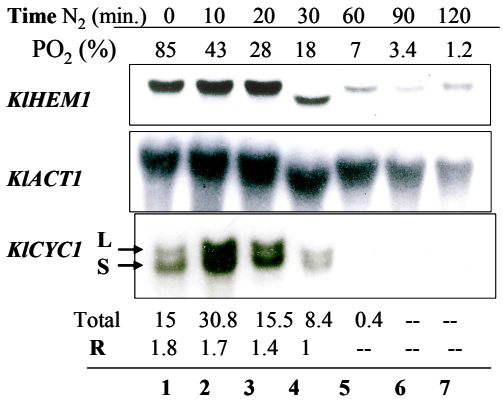

Fig. 3 Stability analysis of *KlCYC1* transcripts under hypoxic conditions. Total: quantitated mRNA signals of the two *KlCYC1* transcripts. R: Ratio between quantitated individual signals of long (L) and short (S) *KlCYC1* transcripts.

We have shown that the UGUR element (position in the *KlCYC1* proximal 3'-UTR) is involved mainly in the stability of the long transcript. The abundance of the short transcript, which also contains this element, is not altered by the mutations, neither in the UGUR sequence nor in the *puf3* mutant.

After a cease in transcription, the two *KlCYC1* transcripts have different stability in the *S. cerevisiae rpb1-1* thermosensitive mutant. Stability for *S. cerevisiae* transcripts goes from 3 minutes (for the less stable) up to more than 90 minutes (for the more stable) [3], allowing us to consider the long *KlCYC1* transcript as an unusually stable transcript when expressed in *S. cerevisiae*. The previous conclusions show the important regulatory role that the proximal 3´-UTR elements plays in the stability of alternative transcripts (including those with the long 3´-UTR), being able to modulate mRNA stability at a distance.

The shift to hypoxia, in a *K. lactis* background, showed rapid degradation for both transcripts under this physiological condition. Both transcripts are degraded faster than in the experiments performed in the *S. cerevisiae rpb1 ts* mutant. The data presented suggest different mRNA turnover mechanisms able to operate on *KlCYC1* transcripts under different physiological conditions and cellular backgrounds. For further applications in heterologous gene expression, the complete *KlCYC1* 3´-UTR will stabilise the mRNAs, while the short element will allow a transient and rapidly degraded expression.

Acknowledgements: This work was supported by a grant XUGA: PGIDT06PXIB103086PR. We would like to thank MI. Gonzalez-Siso for setting up the cultures in the fermenter.

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Characterization of Chitinase Gene from a Paca River Bacterium *Chromobacterium violaceum* UCP1489

M. L. R. Bastos Silva[1,5], M. V. Silva[2], C. M. A. Almeida[3], N. S. A. Freitas[4], H. Almeida Burity[3], and G. M. Campos-Takaki[5]

[1]Programa de Pós-Graduação em Ciências Biológicas – UFPE, Brazil;
[2]Departamento de Bioquímica - UFPE, Brazil;
[3]Laboratório de Genoma - IPA, Brazil,
[4]Departamento de Genética – UFRPE, Brazil.
[5]Núcleo de Pesquisas em Ciências Ambientais, UNICAP 50050-590 Recife-Pernambuco, Brazil

Bacterial strain UCP1489 was isolated from Paca River from the coastal area of Pernambuco, Brazil, and identified as a member of the genus *Chromobacterium*. *C. violaceum* strain UCP1489 was grown for 48h in Luria-Bertani (LB) media at 30°C in shaker at 150rpm. Cultures were centrifuged at 13,000Xg for 10 minutes. The *C. violaceum* ChiA gene was amplified using PCR, the chitinase-encoding gene (ChiA), was cloned, and the nucleotide sequence was determined. Similarity searches were performed using the BLAST algorithm at the NCBI server. Multiple sequence alignments were performed using the ClustalW program. The open reading frame (ORF) of ChiA (carbohydrate transport and metabolism) encoded a protein of 311 amino acids with a calculated molecular mass of 32,625Da. ChiA consisted of only a catalytic domain and showed a significant homology with family 18 chitinases. The N-terminal domain (391 amino acids), this domain is found in a number of bacterial chitinases. It is organized into a fibronectin III module domain-like fold, comprising only beta strands. Its function is not known, but it may be involved in interaction with the enzyme substrate, chitin. It is separated by a hinge region from the catalytic domain (pfam00704); this hinge region is probably mobile, allowing the N-terminal domain to have different relative positions in solution. Was similar this strain UCP1489 and furthermore showed significant sequence homology to the regions CDD:33272 the strain of *C. violaceum,* ATCC 12472, selected from a variety of chitin-utilizing bacterial species as the most active in chitin degradation, has previously been shown to grow on crystalline or colloidal chitin as its sole carbon and nitrogen source.

Keywords chitinase, *Chromobacterium violaceum,* comparative analysis

Introduction

Chromobacterium violaceum is classified as a gram-negative and free-living β-proteobacteria (Neisseriales, Neisseriaceae). *C. violaceum* was defined as a non-pathogenic bacterium that could act as opportunist pathogen, since there are more than 150 reports of cases of septicemia in humans [1]. Although the incidence of these infections in humans is low, the mortality rate is high. Analyses in infected patients in the United States evidenced low pathogenicity of the micro-organism, which causes severe infections, mainly in immunodepressed organisms. Nevertheless, the data available to date are questionable since there is one report of infection of a non-immunocompromised individual [2].

The bacterium can commonly be isolated from water samples, mainly of blackwater rivers, and from soil samples of tropical and subtropical regions of diverse continents. A main characteristic is the purple colour of the colonies due to the pigment violacein. This pigment exhibits antibiotic activity against *Trypanossoma,* causal agent of the Chagas' disease [3] and against *Leishmania* [4].

Living in environments with low nutrient content and low pH, this micro-organism can be considered an extremophile. This kind of organism is extremely interesting for being able to produce enzymes that act in the extreme conditions the micro-organism survives in. Such enzymes can be used for various purposes, as for example in industrial processes, agricultural substances, molecular biology and genetic engineering. In view of the great biotechnological potential, Brazil was one of the countries that took interest in the DNA of this micro-organism. *C. violaceum* was chosen as model for a complete sequencing of the genome structure by the Brazilian National Genome Project Consortium (BrGene), [5].

The data obtained at the end of the sequencing provided a map with the possible genes, distributed in a single circular chromosome of 4.751Kb with high C+G content (64.83%). The number of ORFs (Open reading frames) was 4.431 (average size of 945 pb), which cover 89% of the genome. Of these, 2.717 (61%) were homologous to known proteins, according to data available in the databank (*www.brgene.lncc.br/cviolaceum*). Approximately one third of the identified protein families are related to transport functions while the function of one fourth is yet unknown [5].

The phylogenetic similarity of the sequenced line was 17.4% with *Ralstonia solanacearum*, 9.75% with *Neisseria meningitidis* serotype A and 9.61% with *Pseudomonas aeruginosa*. The highest similarities with *R. solanacearum* were observed in gene ORFs related to environmental interactions, post-translational modifications, cell motility, inorganic ion transport, and secondary metabolite biosynthesis. About 50% of these ORFs are absent from *N.meningitidis.* Since *C. violaceum* and *R. solanacearum* are free-living bacteria, these data suggest that environmental adaptation is linked to the presence or absence of certain gene products [5].

The genomic information available allows for advanced studies that explore the gene functions, regulation and role in a particular metabolic path of interest. Post-genomic studies that investigate the physiology of *C.violaceum* are of great importance, since the biotechnological potential of the products of some genes is highly promising. The objective of this study was to evaluate the corresponding gene product as ORF (Cv2935) from chitinase.

Materials and Methods

Bacterial growth conditions

The lines of *Chromobacterium violaceum* ATCC12472 used as reference in all analyses, were provided by Embrapa Soja, Londrina, PR, Brazil and line UCP1489 was obtained from the culture collection of NPCIAMB (Núcleo de Pesquisas em Ciências Ambientais - UNICAP) and grown in Luria–Bertani (LB) medium [6]. For pre-inoculation, one bacteria colony was transferred to tubes containing 5 mL LB medium and incubated with agitation (150 rpm), at 30^0C for 24 hours at 150 rpm. Thereafter, 250mL Erlenmeyer flasks containing 50mL LB medium were inoculated with 1 mL of each culture and incubated with agitation (150 rpm) at 30^0C for 48h.

Genomic DNA extraction from *C. violaceum*

After cultivation, the cells were centrifuged (13.000 x g) at 4^0C for 10 minutes, the supernatant was discarded, and the cells were washed with 50 µL isotonic solution. The cells were then lysed and the precipitate was extracted with one volume of chloroform/isoamilic alcohol (24:1) under centrifugation as before, and the organic phase discarded. DNA was precipitated with ammonium acetate (7.5 M) and dissolved in TE buffer, quantified on 1.0% agarose gel, and stained with Sybr Gold 1X (Invitrogen Brazil). Horizontal electrophoresis was run using 0.5X TBE buffer for 50 min at 80 V. The gel was visualized on a UV-transilluminator and photographed.

Primer construction

The ORF sequences (likely protein-coding regions) of the possible genes were identified in the Instituto de Genômica Virtual, where the research results of the Projeto Genome Nacional are deposited *(www.brgene.lncc.br/cviolaceum*). The accession number of the ORF sequences used for primer construction Cv2935 (Chitinase A), using software "Primers 3" (http://frodo.wi.mit.edu/cgi-bin/*primers*3/*primers*3_www.cgi).

DNA amplification

DNA was amplified by polymerase chain reaction (PCR). The reaction system contained 30 ng DNA, 10 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 100 µM of each deoxyribonucleoside triphosphate (dNTPs), 0.2 µM the primers 5'- GGCATAGACCTGGATCTGGA -3' (Quit A-L), 5'- CAGTTGATCGACCACGTCAT -3' (Quit A-R), 1.5 U Taq DNA polymerase enzyme (Invitrogen, Brazil) and Mili-Q water of 25µL. Analyses were performed in an MJ Research Inc. PTC-100 thermocycler, and the cycles were as follows: an initial cycle of denaturation at 94^0C for 5 min; 35 cycles of denaturation at 94^0C for 1 min, annealing at 60^0C for 1 min and extension at 72^0C for 2 min; a final extension cycle of 72^0C for 5 min; and a final soak at 4^0C. The PCR products were visualized after electrophoresis at 80V on 1.5% agarose gel. The samples were applied on the gel and stained with Sybr Gold 1X (Invitrogen Brasil), UV-visualized and photographed. A 1 kb Plus DNA ladder was used as molecular weight marker.

Sequencing and sequence analysis

The fragments were sequenced on an ABI 3100 automated DNA sequencer. To confirm the PCR products as members of the chitinase family the sequences were analyzed using Gene Runner 3.01 (www.generunner.com)

to obtain the respective frames and the amino acid sequences were searched by BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), to identify the products and their respective conserved domains (CDD). The sequences were subjected to blastp/NCBI to identify the phylogenetic history.

For the alignment of chitinase A, non-redundant sequences with highest identities of proteins classified as chitinases were chosen in the NCBI. The multiple sequence was aligned using ClustalW 1.8 [7] incorporated in the programme BioEdit [8]. Programme MEGA4 [9] was used for the phylogenetic tree and UPGMA analysis and for a 500-replication bootstrap analysis.

Results and Discussion

For the alignments of chitinase A (exochitinase) using CLUSTAL W, the amino acid sequences of *C. violaceum* ATCC12472 (Embrapa-CNPSO) and UCP1489 obtained by programme Gene Runner 3.01 (www.generunner.com), were also added to the FASTA files (Figure 1).

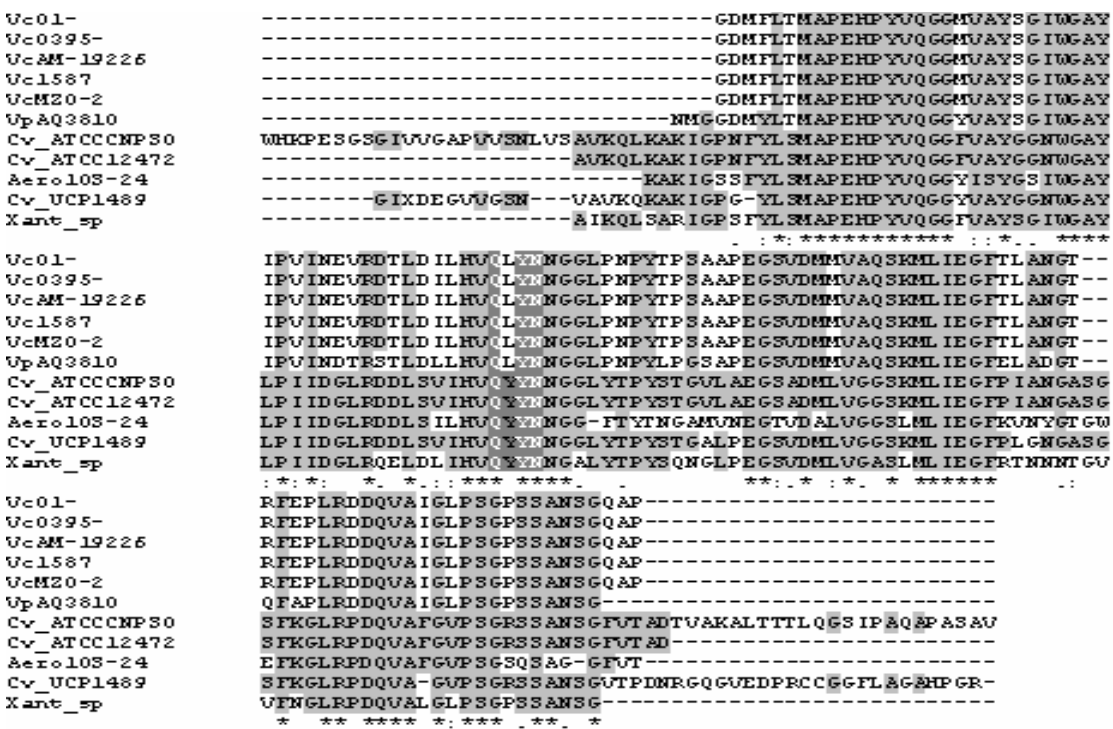

Figure 1: Alignment many of the amino acid sequences of chitinase A of strains of *Chromobacterium violaceum* UCP1489 (UNICAP); *C. violaceum* ATCC 12472 (BrGene); *C. violaceum* ATCC12472 (Embrapa - CNPSO); *Xanthomonas* sp. AK; *Aeromonas* sp. 10 S-24; *Vibrio cholerae* MZO-2; *Vibrio cholerae* AM-19226; *Vibrio cholerae* 1587; *Vibrio cholerae* O1 biovar eltor str. N16961; *Vibrio cholerae* O395; *Vibrio parahaemolyticus* AQ3810, obtained from the program ClustalW. Sites in gray are synonymous to the *Chromobacterium violaceum* and the letters highlighted are the strands β 6 of family 18 of glicosil hydrolase.

Blast revealed the following identity percentages for UCP1489: 96% with *Chromobacterium violaceum* ATCC 12472 (GenBank, accession no. AE016825.1), 74% for *Xanthomonas* sp. AK (GenBank, accession no. BAA36460.1), 70% *Aeromonas* sp. 10S-24 (GenBank, accession no. 2206304C) and 65% *Vibrio cholerae* MZO-2 (GenBank, accession no. EDM54037.1), 65% *Vibrio cholerae* AM-19226 (GenBank, accession no.EDN14982.1), 65% *Vibrio cholerae* 1587 (GenBank, accession no. EAY32809.1), 65% *Vibrio cholerae* O1 biovar eltor str. N16961 (GenBank, accession no.AAF95100.1), 65% *Vibrio cholerae* O395 (GenBank, accession no. ABQ21983.1) and 65% *Vibrio parahaemolyticus* AQ3810 (GenBank, accession no. EDM60703.1), 65% *Aeromonas salmonicida* subsp. salmonicida A449 and *Aeromonas hydrophila* subsp. hydrophila ATCC.

The multiple alignments of homologues for chitinase A of UCP1489 revealed 172 sites, of which 65 were conserved, while among the *Chromobacterium* lines the number of bases of synonymous amino acids was 118 sites. The *C. violaceum* UCP1489 sequence exhibited coincidence of alignment of 227 amino acids with the N-terminal of sequence AAQ60603.1, which corresponds to product CV2935 and is described as a likely chitinase

in the Cv ATCC 12472 genome (BrGene), consisting of 439 amino acids. There are two regions for this product: i) a ChtBD3 is a key domain for chitin type 3 (29..73) and corresponds to CDD:47799; ii) a chitinase A with CDD: 33272 that is responsible for carbohydrate transport and metabolism (109..419) and belongs to glycosyl hydrolase family 18. The N-terminal domain of this chitinase A was already found in free-living bacteria [10] and the mean consensus (region of amino acid sequence in grey, Figure 1) with the other chitinases was 118 amino acids and corresponded to the domain that characterizes the functionality of the enzyme chitinase of line UCP 1489 (Figure 2).

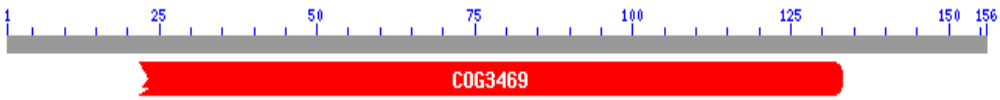

Figure 2: Domain of COG3469 enzyme chitinase the family 18 of glicosil hydrolase for the lineage UCP 1489 according to the result Blast - PDB. The figures in blue and represents the residues of amino acids.

Glycosyl hydrolase family 18 (GH18) of chitinase is an old gene family that is widely expressed in archaea, prokaryotes and eukaryotes. Certain bacteria and plant groups do not produce chitin and chitinases, respectively, for the conversion of insoluble chitin into metabolizable nutrient sources and for the chitins of pathogen defence [11]. The highlighted letters In Figure 3A indicate the characteristic β-6 strands of glycosyl hydrolase family 18 (QxYN) that are part of the chitinases, which can exhibit β 1-8 in chitinase B [12].

Phylogenetic tree constructed with the homologues of UCP 1489, with identity > 60%, using amino acid sequences of the enzyme chitinase of the of the glycosyl hydrolase family 18 deposited/implemented in the GenBank, formed two groups. Group I consisted of chitinase of *Vibrio*, *Chromobacterium* and *Aeromonas*. Group II consisted of four bacteria of three genera *Vibrio (*Figure 3).

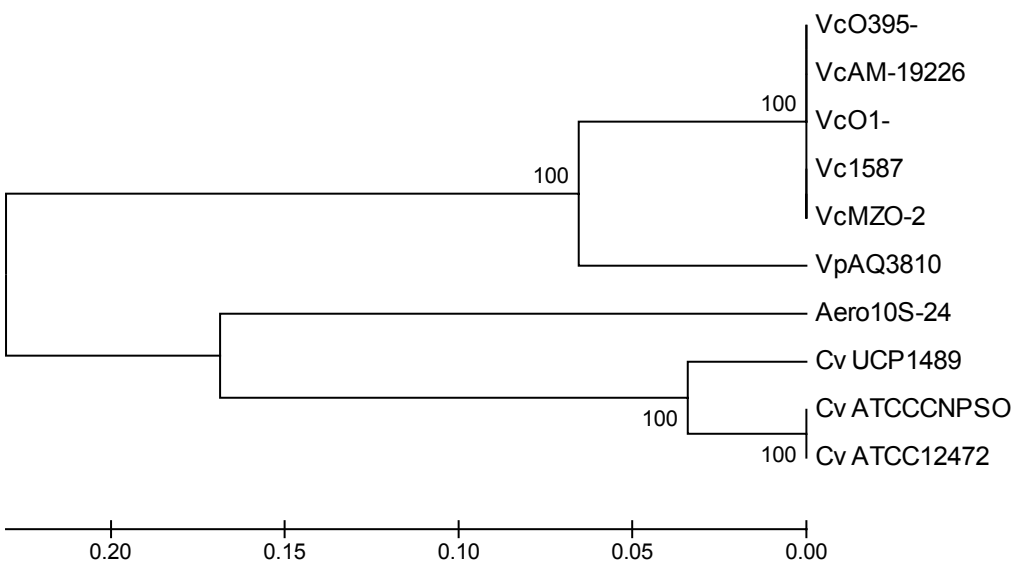

Figure 3: Tree of sequences of amino acids of the strains of *Chromobacterium violaceum* ATCC 12472 (BrGene); *C. violaceum* ATCC12472 (Embrapa - CNPSO); *C. violaceum* UCP1489 (UNICAP); Aeromonas sp. 10 S-24; *Vibrio cholerae* MZO-2; *Vibrio cholerae* AM-19226; *Vibrio cholerae* 1587; *Vibrio cholerae* O1 biovar eltor str. N16961; *Vibrio cholerae* O395; *Vibrio parahaemolyticus* AQ3810 corresponding to the region COG3469 of chitinase, obtained by test phylogenetic Bootstrap as Neighor - Joining, with 500 repetitions in UPGMA.

The alignment region of chitinase A with highest number of non-synonymous amino acid sequences with the *C. violaceum* lines was the central region of *Aeromonas* sp. 10S-24 with 17 bases [13]. But this is the only bacteria genus with identity > 60% for the two chitinase sequences. The greatest variations of the initial and final regions were verified intra-specifically in *C. violaceum* (Figure 1, bases without grey background).

Conclusions

Was similar this strain UCP1489 and furthermore showed significant sequence homology to the regions CDD:33272 the strain of *C. violaceum,* ATCC 12472, selected from a variety of chitin-utilizing bacterial species as the most active in chitin degradation, has previously been shown to grow on crystalline or colloidal chitin as its sole carbon and nitrogen source.

Acknowledgements: CNPq, CAPES, FINEP for financial support, and IPA, UNICAP for facilities.

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Characterization of *KlHIS4* transcriptional regulation by growth-media nutrients

M. Lamas-Maceiras*, A. M. Rodríguez-Torres and M. A. Freire-Picos

Facultad de Ciencias, Universidade da Coruña. Campus da Zapateira S/N 15071 A Coruña, Spain.

*Corresponding author: M. Lamas-Maceiras. mlamas@udc.es. Phone: +34 981167000

The gene *HIS4* from *Kluyveromyces lactis* is transcriptionally activated in complete synthetic respect to rich media and in an independent mechanism related to carbon source. This regulation was not previously described for *Saccharomyces cerevisiae HIS4.* The EMSA assay carried out with F7 showed a specific band, Fc1, in YPG, and two bands, Fc2 and Fc3, in complete medium. The Fc2 and Fc3 bands were dependent on the carbon source present in the medium, since their intensities were higher in glycerol than in glucose. The protein or proteins causing the Fc1 band seem to be involved in the different regulation mechanisms between rich and synthetic complete media because the Fc1 band was detected in cells grown in synthetic medium. Therefore, the promoter region (-200 to -173) is responsible for two independent regulatory mechanisms.

Keywords transcriptional regulation; carbon source; yeast; *Kluyveromyces lactis*.

1. Introduction

The transcriptional regulation of *Saccharomyces cerevisiae HIS4* gene, involved in the histidine biosynthetic pathway, is mainly carried out under two mechanisms, General Control mediated by Gcn4 transcription factor [1], ensuring activation during amino acid starvation, and Basal Control mediated by Bas1 and Bas2 factors when there is no amino acid starvation [2]. Despite the high degree of homology between *HIS4* and *Kluyveromyces lactis HIS4* (*KlHIS4*) and the presence of a putative Bas1 and Bas2 and several Gcn4 binding sites in the *KlHIS4* promoter, the transcriptional regulation of this gene is not mediated by General or Basal Control mechanisms. Furthermore, and in contrast to what is described for *HIS4*, *KlHIS4* is transcriptionally activated by ammonia starvation and oxidative stress. Analyzing the sequence of *KlHIS4* promoter, several putative binding sites to factors related to these kinds of transcriptional regulation, Nit2 and Ap1, were found [3].

Interestingly, there was transcriptional activation of *KlHIS4* in complete synthetic versus rich media and YPG (2% glycerol) respect to YPD (2% glucose) (Fig. 1). The *KlHIS4* promoter sequence presented a putative Hap2/3/4 binding site related to transcriptional activation in the presence of non-fermentable carbon source. This regulation was not previously described for *HIS4.*

Knowledge of the *KlHIS4* transcription regulation will be useful in the development and optimization of biotechnological applications using *Kluyveromyces* strains. In this work, we present the characterization of the promoter region responsible for transcriptional regulation by carbon source and synthetic complete versus rich media.

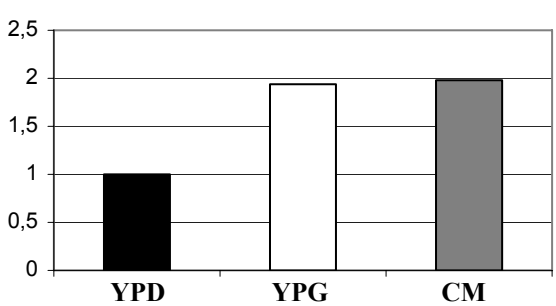

Fig. 1 Data of normalized transcript signal, average from five independent experiments. To compare expression in the different media, expression in YPD media was given the value 1, in arbitrary units, and it was used as reference for the other signals.

2. Materials and Methods

2.1. Yeast strain and growth conditions

Kluyveromyces lactis wild type strain NRRL-Y1140 (*MAT a,* from Zitomer, R.) was used in Electrophoretic Mobility Shift Assays (EMSA). In these experiments, cells were incubated in yeast synthetic complete medium (CM) (containing 2% glucose) and rich media, YPD or YPG (containing 2% glucose or glycerol, respectively) [4].

2.2. Electrophoretic Mobility Shift Assays (EMSA)

Extracts and Gel Retardation assays were performed as in [5]. The protein extract concentration was determined by the Bradford Method [6]. EMSA assays were carried out with the two *KlHIS4* promoter fragments obtained, after annealing specific oligonucleotides. Fragment 6 (F6) includes the promoter region -236 to -200, oligos: oMLPR18: 5'-TTACTTCTTCTTCGAGTTTTCATTGGCTTTCCTGTGT TTTTTTAT-3' and oMLPR19: 5'-TTTATGAAAAAAACACAGGAAAGCCAATGAAAACTCGAAG AAGAAGT-3'. Fragment 7 (F7) includes the region -200 to -173, oligos: oMLPR22: 5'-TTTTCATCA TATGACTCATGTAATTC-3' oMLPR23 5'-TTTGAATTACATGAGTCATATGATGA-3'. The fragments were labelled by Klenow and α-32P-ATP. The binding reactions were carried out at 30 ºC for 20 minutes in buffer A [5] with 10000 cpm of labelled DNA, 15 μg of protein extract and 2 μg of calf thymus DNA as carrier. In competition experiments, double amount of unlabelled F7 was used as specific competitor.

3. Results and Discussion

3.1. Search of putative binding sites in the delimited promoter region responsible for the regulation of growth-media nutrients

A previous characterization of *KlHIS4* promoter delimited a promoter region, encompassing positions -236 to -173, (F5) (Fig. 2a) as responsible for the regulation by carbon source and synthetic complete versus rich media. In this region, two putative Ap1/Gcn4 and one Hap2/3/4 binding sites were found.

To analyze by EMSA the regulatory elements responsible for this regulation depending on media conditions, the promoter region indicated in Fragment 5 was split into two fragments for further characterizations. Fragment 6, encompassing positions -236 to -200, included putative Hap2/3/4 and Ap1/Gcn4 binding sites, and Fragment 7 encompassed positions -200 to -173 (Fig. 2a).

3.2. Fragment 6 is not involved in regulation by growth-media nutrients

The EMSA assay was performed with F6 to test if this promoter fragment was involved in the transcriptional activation of *KlHIS4* previously detected in glycerol by Northern blot. Although there was a putative Hap2/3/4 binding site in the sequence of this fragment, the faint bands observed did not present differences between the conditions tested (Fig. 2b, lanes 2 and 3), indicating that F6 is not counting for these two regularory mechanisms

3.3. Fragment 7 contains the elements for growth media dependent regulation of *KlHIS4*

The EMSA assay carried out with F7 showed the presence of a specific band, Fc1, in CM (Fig. 2b, lane 6) and two bands, Fc2 and Fc3, in YPG (Fig. 2b, lane 7). Their specificities were tested using two-fold excess of F7 non-labelled fragment as specific competitor. The intensities of the three bands shown in Fig. 2b are diminished in the presence of specific competitor (compare lanes 10 and 11, and 12 and 13).

The Fc1 band is caused by a protein or proteins involved in the regulation between rich and synthetic media because it is detected in cells grown in synthetic complete medium containing glucose but not in YPD (Fig. 2b, compare lanes 6 and 8).

In rich medium containing glycerol, two bands, Fc2 and Fc3, were observed. These bands are dependent on the carbon source, since they appear in rich medium containing 2% glycerol but not in 2% glucose (Fig. 2b, compare lane 7 and 8). The Fc2 band can be observed in CM but its intensity is clearly higher in YPG (Fig. 2b, compare lanes 10 and 12).

Olesen and co-workers found notable differences in amino acid concentrations in rich and synthetic media [7]. However, Northern experiments performed in our laboratory indicated that the amino acid concentration is not causing the transcriptional activation of *KlHIS4* in synthetic complete medium (data not shown).

These results indicate that the promoter region -200 to -173 (F7) is the region involved in the regulation of *KlHIS4* by carbon source and synthetic versus rich media. It is interesting to note that this region is responsible for two independent regulatory mechanisms, since the Fc1 band is clearly caused by the protein or proteins binding in response to differences in nutrient composition between synthetic complete and rich media while Fc2 and Fc3 are caused by factors whose binding activities respond specifically to the carbon source.

These two regulatory mechanisms are not exclusive to *KlHIS4* since previous analyses of other *K. lactis* genes showed that *KlLEU2* and *KlTRP1* are also under regulation by carbon source. However, only *KlLEU,* as *KlHIS4,* has different mRNA levels in cells grown in synthetic complete and rich media [3].

These data reinforce previous results [3, 5] regarding a different carbon source dependent regulation of *K. lactis* genes in contrast to *S. cerevisiae* and open the field to study new promoter regulatory elements and proteins that may cause these two independent promoter regulation responses.

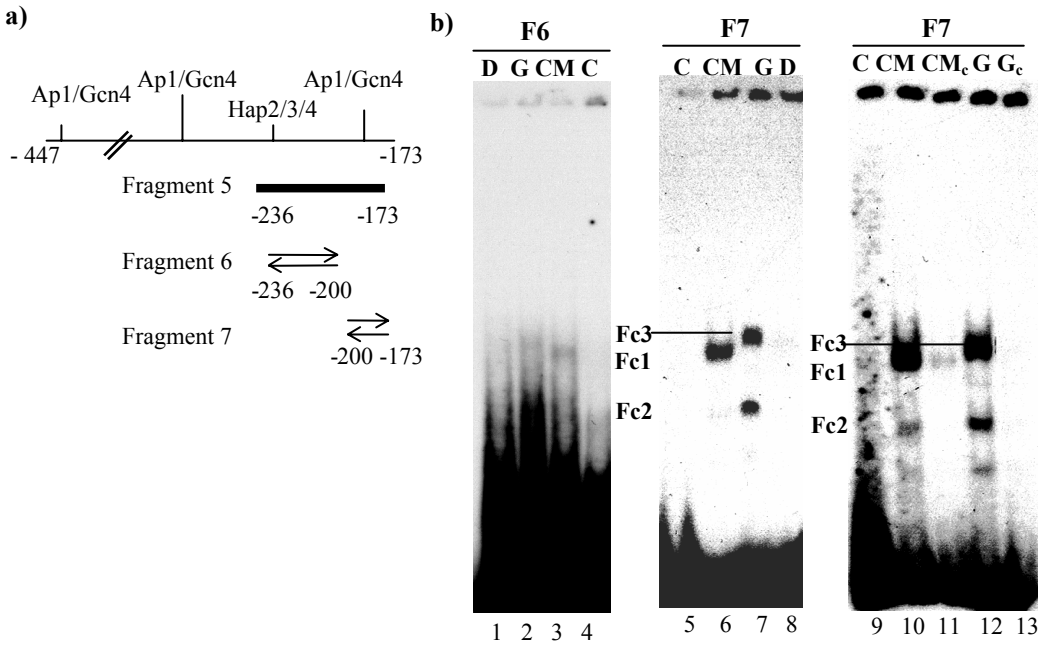

Fig. 2 a) Diagram of *KlHIS4* promoter showing the putative binding site. The fragment of *KlHIS4* promoter obtained by PCR is shown as a dark bar (F5) and the fragments obtained by oligonucleotides annealing as a double arrow (F6 and F7). **b)** EMSA assay using F6 and F7. Lanes 9 to 13, **c**ompetition EMSA assays with two-fold excess of specific competitor, non-labelled F7. C: reaction control without protein extract, CM: synthetic complete medium containing glucose, D: YPD, G: YPG, c: specific competitor.

Acknowledgements The support by XUGA PGIDTO6PXB103086PR and 1030lB98 is gratefully acknowledged.

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Desulfovibrio vulgaris Hildenborough transcriptomic analysis by Restriction fragment functional display (RFFD)

M. Santana and J. González*

Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

*Corresponding author: Tel./fax:+ 34 954624711/002, e-mail:jmgrau @irnase.csic.es

Sulfate-reducing bacteria (SRB) reduce sulfate with electrons from carbon substrate thereby producing hydrogen sulfide. This reduction is anaerobic, however the metabolic activity of SRB in oxic zones is frequently higher than in neighbor anoxic zones. The large tolerance to oxygen of SRB is surprising; some are able to respire oxygen in a process coupled to chemiosmotic conservation of energy and ATP production. In the case of *Desulfovibrio vulgaris* Hildenborough sequencing of its genome has confirmed the presence of putative oxygen reductase genes responsible for oxygen consumption. We propose a comparative study of *D. vulgaris* Hildenborough gene expression under aerobic and anaerobic conditions in order to understand the mechanism of the response to oxygen in this SRB. Restriction Fragment Functional Display (RFFD) technique, here described, constitutes a powerful tool to do such study.

Keywords Desulfovibrio vulgaris Hildenborough; Restriction Fragment Functional Display; response to oxygen

1. Background

In SRB the electrons necessary for sulfate reduction are provided by oxidation of organic molecules by periplasmic or membrane-bound enzymes, several of these enzymes are oxygen sensitive [1, 2, 3]. In addition, the chemical reaction of oxygen with sulfate-reducers reduced compounds produce super oxide radicals, which are toxic to the cells [4]. The presence of detoxifying enzymes super oxide reductase [5, 6], NAD(P)H-dependent peroxidases such as ruberythrins [7,8], super oxide dismutase [9, 10] and catalase [9, 11], these last two typical of aerobic micro organisms, accounts for the ability of sulfate-reducing bacteria to survive for long periods of exposure to high levels of oxygen.

Several SRB are even able to use oxygen as an electron acceptor reducing it to water [12], and aerobic respiration can supports chemiosmotic energy conservation [13]. Despite this energy-coupled oxygen reduction, aerobic growth of sulfate reducing bacteria is poor or absent [14], the only example of oxygen-dependent growth at nearly atmospheric levels was recently reported for *D. desulfuricans* ATCC 27774 [15].

Genes coding for terminal membrane oxygen reductases have been found in SRB. *D. vulgaris* Hildenborough and *D.desulfuricans* G20 possess cytochrome *c* oxidase and cytochrome *bd* oxidase genes [11, 16]; this last enzyme is homologous to a high-affinity cytochrome *bd* oxidase isolated from *D.gigas.* [17]. The oxygen consumption rate of a *D. vulgaris* cytochrome *bd* oxidase deletion mutant is about 30% of the wild-type rate, indicating that the enzyme functions as an oxygen reductase in vivo (M. Santana, unpublished data).

Profiting from the availability of *D. vulgaris* Hildenborough genome sequence, we propose a differential analysis of genome-wide gene expression by Restriction fragment functional display (RFFD) in order to assess the mechanisms implicated in the response to oxygen in this microorganism.

2. Methodology

DNA micro arrays are widely use in transcriptomic analysis. However, micro-arrays are a "closed" system for gene discovery, where only those known genes spotted on a chip will be analyzed. Moreover, DNA micro arrays involve hybridization of complex mRNA probes as single-stranded, non-uniformly labeled cDNAs, yielding dubious results on genes with low expression levels or similar paralogs, a consequence of cross-hybridization [18]. In contrast, Differential display (DD) does not require any prior knowledge on specific mRNA sequences making the gene screening unbiased. In addition, DD visualizes the mRNA in subsets directly after their amplification and labeling, thus being capable of detecting both known and novel genes for any organism. Differentially expressed cDNA bands can be further cloned and sequenced. The Restriction fragment functional display (RFDD) technique here described is a modification of the Restriction fragment differential display (RFDD) technique [19, 20] that profits from the knowledge of total genome sequence, thus avoiding the need of additional reamplification and sequencing in traditional DD. Briefly, after extraction of total RNA from cells

growing under defined conditions, synthesis of double-stranded cDNA is made by reverse transcription with random hexamers. The cDNA is digested to fragments of *ca* 200 bp and adaptors are ligated to those fragments, which are amplified by PCR using 5' fluorescently labeled adaptor primers, each primer with different 3'-dinucleotide sequences. The amplified products are separated in a sequencer and computer analysis of fragments of different length and intensity is made. Based on the length and flanking sequences of the fragments, these are identified using a restriction enzyme fragment database, conceived from the annotated genome sequence.

In order to use RFFD several parameters have to be established. Hexamer sequences have to be designed so that synthesis of cDNA is selected discriminating against rRNA. Table I shows the hexamers selected for *D. vulgaris* Hildenborough, designed from inspecting every possible combination of bases in its genome sequence. The ten sequences in the table do not amplify rRNA genes since they are not complementary to any of them. Combining the first six sequences as a set of non-random hexamers, result in priming the mRNA for reverse transcription reactions every 140 bp, on average. (Fig 1). Among several restriction enzymes of potential use in *D. vulgaris* Hildenborough RFFD analyses, TaqI was chosen since average fragment length after digestion is 146,6 bp and the percentage of fragments between 50 and 500 bp is 65.9.

For transcriptomic analysis *D. vulgaris* Hildenborough wild-type and Δ*bd* (a deletion mutant of cytochrome *bd* oxidase genes) cells were grown in anaerobiosis in a lactate-sulfate medium [21] until mid-exponential phase [OD600 nm 0.5-0.7], and then subjected to aeration *ca.* 12 hours. RFFD was then performed on RNA extracted from both mid-exponential anaerobic and aerated cultures.

Table I. Distance in bp between *D. vulgaris* Hildenborough genome's initiation sites for cDNA synthesis.

Target No.	Primer sequence	Cumulative average fragment length	Percentage of fragments <300 bp.	Percentage of fragments ≥300 bp.
1	**NCATCA**	567.8	47.45	52.55
2	**NTGGGG**	343.6	60.32	39.68
3	**NGTGAA**	248.5	71.07	28.93
4	**NAAGCG**	194.6	78.60	21.40
5	**NCAAGA**	159.4	84.12	15.88
6	**NTTGTC**	136.6	87.77	12.23
7	NAACAT	122.9	89.79	10.21
8	NAAAGC	112.1	91.24	8.76
9	NAATGC	101.0	92.79	7.21
10	NAGTGG	92.1	93.90	6.10

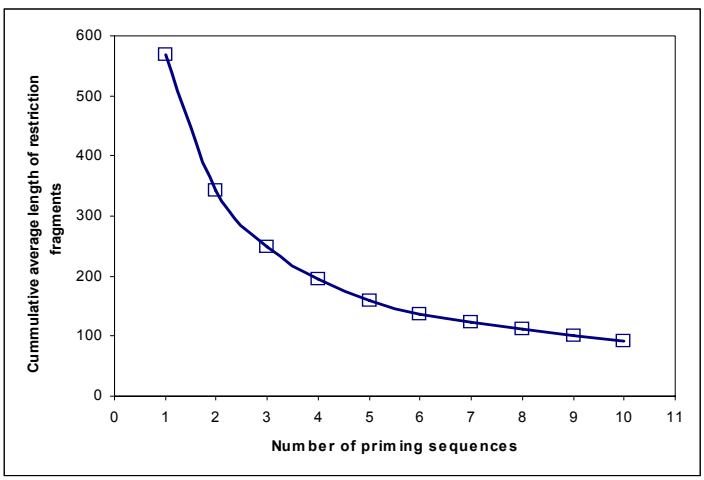

Fig 1. Distance in bp between priming sites in the genome of *D. vulgaris* adding each primer subsequently; a mixture of the sequences 1 to 6 (see Table I) allows the synthesis of fragments of *ca* 140 bp.

3. Results and discussion

3.1 Performance of RFFD methodology

Previous reports on *D. vulgaris* transcriptomic analysis by DNA microarrays gave limited results, only a few hundred genes were identified as up or down-regulated out of thousands in a genome [22]. Our RFFD analysis identified over 1000 genes showing differential expression (see section 3.2). For comparisons where the same amplified restriction fragments are found in both conditions, the calculation of the frequency distribution of the Log_2 ratios was done. From that calculation we estimated that over 95% of data were within 1.5 Log_2 units around the average, meaning that data points with an expression factor of 2.8 or more can be considered significant, a factor of the same magnitude as compared to those obtained using other techniques.

3.2 Genes associated with oxygen response

Aeration of the cultures didn't result in a significant change of the growth curves. This survival behavior can be the result of an increase in transcription of the genes encoding detoxifying enzymes, Indeed, low height peaks where detected both in wild-type and bd mutant in oxygenated conditions, corresponding to genes codifying super oxide dismutase and a rubredoxin-like protein, the electron transfer partner of ruberythrin. Previous works, enclosing DNA array analysis, also report an increase of peroxide stress response genes together with a mild response in cell viability and mRNA changes when cells are exposed to low-levels of aeration [22]. A low level of aeration also occurred in our experiments, where dissolution of air in the culture flasks was limited by sulfides and moderate agitation (60 rpm at 30ºC). Nevertheless, when considering the fragments detected in only one strain and one growth condition more than thousand open reading frames (ORF) are identified. For those ORFs, the level of differential expression has to be determined by Real-time PCR.

When comparing the expression level (Log10 peak height) for each strain under anaerobic and oxygenated conditions and for different strains under similar conditions it is notorious the existence of a differential gene expression for the mutant under oxygenated conditions (Fig 2 and Table II).

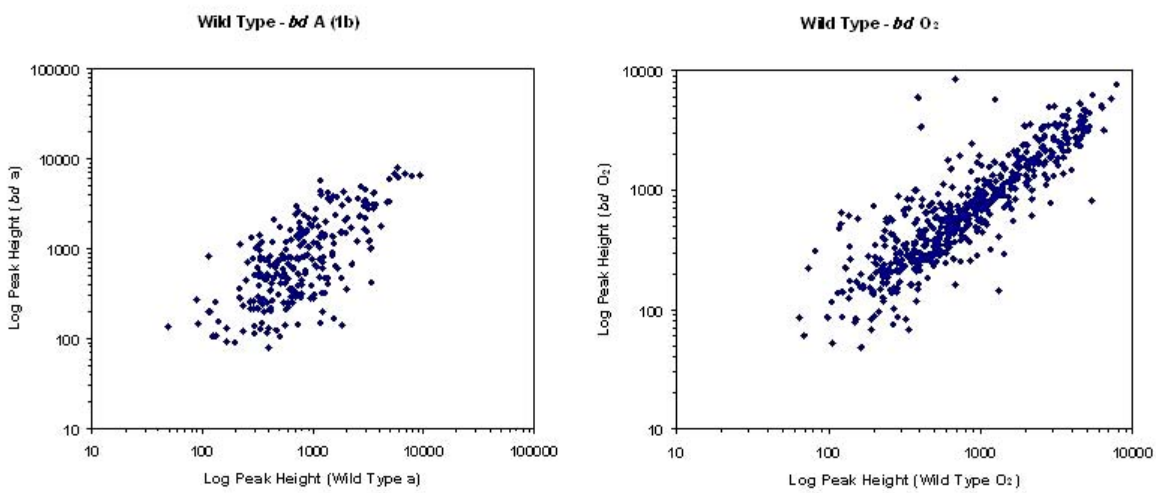

Fig 2. Comparison of the expression level between strains under anaerobiosis (A) and under aeration (O_2). Only the fragments that were detected in both experiments are plotted.

Given the performance of this technology, further RFFD analysis will be performed, in particular using controlled conditions of aeration. In addition, complementary studies to determine the nature of the identified transcriptional regulatory proteins, involving cloning and mutant complementation tests are being planned.

Table II. Up regulated open reading frames (ORF) in the deletion mutant Δ*bd.*

NCBI* ORF annotation	ORF
DVU1623	**CTP synthase (pyrG)**
DVU1300	**Translation elongation factor G (fusA-1)**
DVU1548	**Outer membrane transport protein, OmpP1-FadL-Todx family**
DVU1606	**Potasium uptake protein, TrkA family**
DVU0717	**GGDEF domain-BAL domain protein**
DVU1063	**Transcriptional regulatory protein**
DVU1460	**Hypothetical protein**

*National Center for Biotechnology Information

Acknowledgements We thank Professor Gerrit Voordouw for kindly providing Δ*bd* mutant strain from *D. vulgaris* Hildenborough. M. Santana was recipient of a grant (BGCT/11783/2003) from Fundação para a Ciência e Tecnologia, which was associated with the project POCTI/BIO/43085/2001.

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Effect of some redox-mediators on the decolorization of Acid Orange 7 by resting *Rhodococcus erythropolis* and *Alcaligenes faecalis cells*

T. Avramova*, L. Stefanova, B. Angelova, S. Mutafov

Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Acad. G. Bonchev St., Block 26, 1113 Sofia , Bulgaria

*Corresponding author. E-mail: avramovat@yahoo.com

Studied was the effect of the natural (riboflavin, RF) and synthetic (9,10-antraquinone-2, 6-disulphonic acid disodium salt, AQDS and 2-hydroxy-1, 4-naphthochinon, LQ) redox-mediators on the microbial decolorization of the azo dye Acid Orange 7 (AO7) by resting cells of the Gram negative *Alcaligenes faecalis* 6132 and the Gram positive *Rhodococcus erythropolis 24.* Experiments were performed in conditions of limited oxygen supply. Results obtained in the experiments on AO7 decolorization with two different bacterial strains, *Rh. erythropolis* and *A. faecalis*, underlined the fact that the influence of redox-mediators on the decolorization reaction depended not only on the kind of the redox-mediator used but to a higher extent on the type of the microorganism performing the process. Results about the influence of redox-mediators on AO7 decolorization in conditions of limited oxygen supply showed a similarity with those reported in the literature about experiments in strictly anaerobic conditions.

Keywords microbial decolorization, Acid Orange 7, Rhodococcus erythropolis, Alcaligenes faecalis, riboflavin, 9,10-anthraquinone-2,6-disulphonic acid disodim salt, 2-hydroxy-1,4-naphthochinon

1. Introduction

The azo dyes wasting from different branches of the industry are among the most widespread pollutants of water basins. These synthetic compounds are typical xenobiotics due to the presence of azo bonds (−N=N−) in their structure which renders them high resistance to microbial attack. Nevertheless, the microorganisms decolorize azo dyes by reductive split of the azo bond resulting in formation of colourless products.

It is considered that the electron transfer rate is the main factor determining the decolourisation rate [1-3]. In anaerobic conditions decolourization rate could be significantly accelerated by the addition of quinone compounds which act as redox mediators between the reduced cofactors of the microbial unspecific reductases and the azo bond. Thus, the enzymatically generated hydroquinones in a purely chemical reaction reduce the azo bond of the dye [4, 6-8]. Aside the quinones, flavins (FMN, FAD) are common enzyme cofactors, involved in azo dye reduction [1, 3, 5, 9].

This study is dedicated to the effect of riboflavin (RF) as a natural redox mediator and to the effect of two artificial mediators: 9,10-anthraquinone-2,6-disulphonic acid disodim salt (AQDS) and 2-hydroxy-1,4-naphthochinon (LQ) on the decolourisation of the mono-azo dye Acid Orange 7 (AO7; Tropaeolin 000 № 2; 4-(2-hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt). Resting cells of Gram negative (Gm^-) *Alcaligenes faecalis* 6132 and the Gram positive (Gm^+) *Rhodococcus erythropolis* 24 were used for the process of azo-bond reduction.

2. Materials and Methods

The azo dye AO7 was from Fluka Chemie AG (Buchs, Switzerland). The redox mediators AQDS and LQ were from Sigma-Aldrich. All other reagents were of analytical grade.

Alcaligenes faecalis 6132 and *Rhodococcus erythropolis* 24 were cultivated for 24 h at 28 ˚C on a rotary shaker in 200-mL cotton-plugged Erlenmeyer flasks, each containing 50-mL nutrient broth Standard I (Merck AG, Darmstadt, Germany). The cells were harvested by centrifugation, washed and resuspended in a mineral salt solution, pH 7.2, based on a prescription No. 465 of DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany) with omitted sources of N and S.

The bacterial suspension contained 27 g L^{-1} cells calculated as dry mass.

The decolourisation reaction was carried out in 100-mL iodine flasks, each containing 30 mL cell suspension. AO7 was added to final concentration 10 mg L^{-1}.

The redox mediators were added to the reaction medium simultaneously with the AO7 to final concentrations from 0.015 to 0.5 mM. The flasks were plugged with plastic stoppers and incubated statically at 28 ˚C.

A sample of 1 ml was clarified by centrifugation. The residual AO7 was evaluated spectrophotometrically at 483 nm.

3. Results

3.1 Effect of redox mediators on the AO7 decolourisation

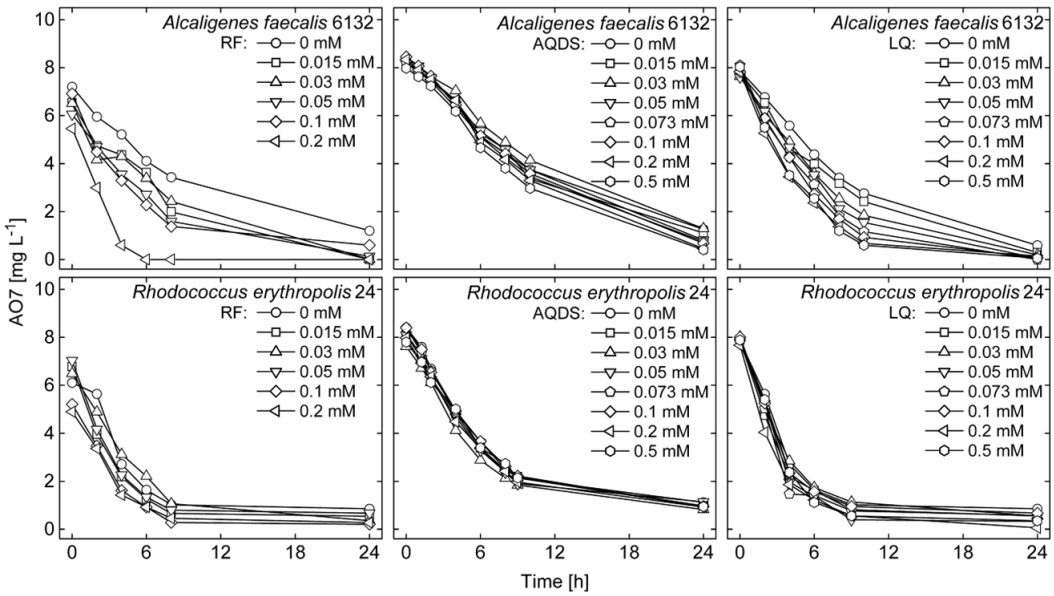

Fig. 1. Dynamics of AO7 decolorization in the presence of RF, AQDS and LQ.

As seen on the graphs (Fig. 2), there are distinct differences in the response of the Gm^- and Gm^+ cells to the presence of the redox-mediators. A minimal positive effect of the synthetic redox-mediator AQDS was observed with the cells of *A. faecalis*, while it had no effect on the process carried out by *Rh. erythropolis* cells. The synthetic redox-mediator LQ affected positively the *Rh. erythropolis* cells and revealed a minimal effect on *A. faecalis* cells. The accelerating effect of RF on the decolorization of AO7 was better manifested with *A. faecalis* cells.

3.2 Effect of tested redox mediators on AO7 decolourisation in presence of glucose as a co-substrate

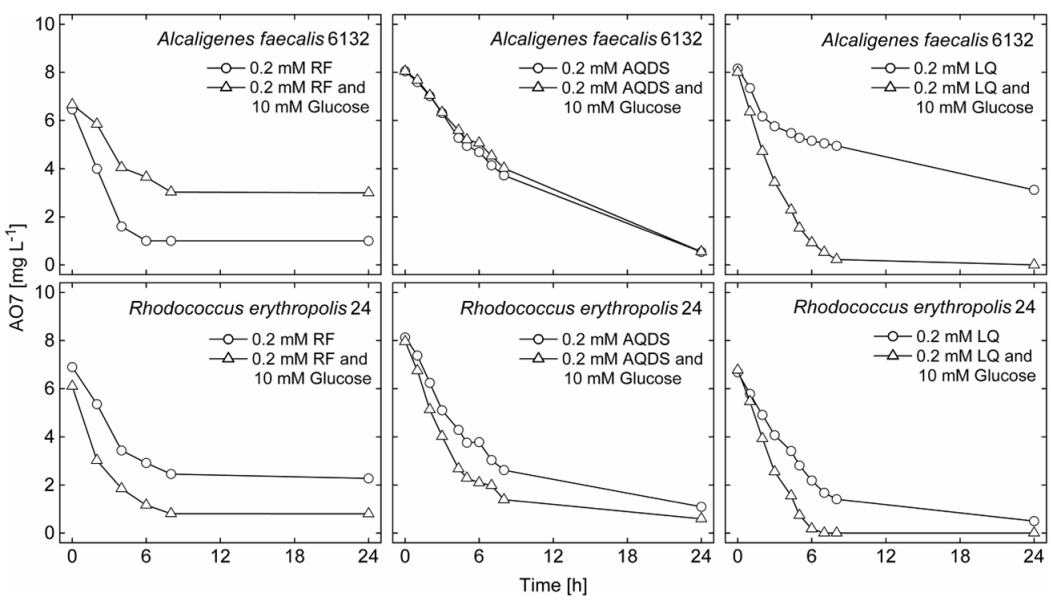

Fig. 2. Dynamics of AO7 decolorization in the presence of RF, AQDS, LQ and glucose as a co-substrate.

Glucose is applied in processes of anaerobic decolorization of azo-dyes as an additional donor of electrons for synthesis of reducing equivalents like NADH, NADPH and FADH, responsible for the activity of various enzyme systems in the microbial cells [5, 7]. Results presented (Fig. 2) reveal the effect of glucose on the decolorization process carried out in conditions of limited oxygen supply. There is a visible beneficial effect of glucose on the decolorization process carried out by Gm^+ cells in presence of AQDS while the combined effect of the glucose and LQ was better manifested with Gm^- cells. Riboflavin in combination with glucose affected negatively the decolorization capacity of the Gm^- *A. faecalis* cells and accelerated to some extent the process carried out by Gm^+ *Rh. erythropolis* cells.

4. Discussion

Results about the influence of redox mediators on decolorization process in conditions of limited oxygen supply showed a similarity with those reported in the literature about experiments in strictly anaerobic conditions [10]. These results confirmed our suggestion that the annaerobic conditions are not obligatory for the decolorization of azo dyes by high-density resting bacterial cell suspension and it could be carried out as well in conditions of limited oxygen supply. Furthermore, the results obtained in the experiments with the two different bacterial strains emphasized (underlined) the fact that the influence of redox mediators on decolorization process depends not only on the kind of the redox mediator used, but to higher extent on the type of the microorganism performing the process. The good decolorization rate of *Rh. erythropolis* control cells and lack of any, or the minimal accelerating effect of the redox mediators supposed, probably, a direct enzyme reduction of the azo-bond of the dye AO7. The relatively high activity manifested by *Rh. erythropolis* control cells makes unnecessary the addition of the redox mediators in the decolourisation process. The beneficial effect of the redox mediators, especially of riboflavin, on AO7 decolorization carried out by *A. faecalis* cells, suggested that a chemical reaction of the azo dye with the enzymatically generated reduced redox mediators takes place and it is responsible for the acceleration of the azo-bond reduction. The alternative response of the bacterial strains to the presence of the redox mediators is, most probably, due to the differences in the cell wall and membranes characteristics of Gm^- *A. faecalis* and Gm^+ *Rh. erythropolis* cells.

Acknowledgements The study was supported by the Foundation for Scientific Investigations of the Bulgarian Ministry of Education, Science and Technology, according to the contract B-1311/2003.

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Heterologous expression and enzymatic characterisation of exopolygalacturonase PGX1

A. de las Heras[1,4], C. Vázquez[2], A. Martínez del Pozo[3], B. Patiño[2], and M. T. González-Jaén[1,*]

[1]Departamento de Genética, [2]Departamento de Microbiología III and [3]Departamento de Bioquímica. Facultad de Biología. Universidad Complutense de Madrid. 28040-Madrid. Spain.
[4]Centro Nacional de Biotecnología Consejo Superior de Investigaciones Científicas (CSIC), Darwin 3, 28049-Madrid, Spain (current address)

[*]Corresponding author: e-mail: tegonja@bio.ucm.es Phone: +34 913 944 830

Fusarium oxysporum f. sp *radicis lycopersi* (FORL) is a tomato pathogen which produces several cell wall degrading enzymes during the process of colonisation. Among these enzymes, polygalacturonases (PGs) are important because of their role in deconstruction the plant cell wall during host plant infection. Production of PGs in heterologous systems is an alternative approach to characterize individual enzymes, a difficult task, since complex patterns of PG isoforms, arising from different genes and patterns of postraductional modifications are generally present in *in vitro* cultures. In this work, we have successfully performed the heterologous expression of one exopolygalacturonase of FORL for the first time in the methylotrophic yeast *Pichia pastoris.* The enzyme produced showed an exo mode of action as predicted, and optimal pH and temperature values of 5 and 55 ºC, respectively. Heterologous PGX1 showed higher affinity for trigalacturonic acid than for digalacturonic or polygalacturonic acid. The enzymatic features analyzed are discussed in relation to the other exopolygalacturonase (PGX2) described in *F. oxysporum* f.sp. *radicis lycopersici.*

Keywords Exopolygalacturonase; Heterologous expression; *Fusarium oxysporum* f. sp. *radicis lycopersici; Pichia pastoris.*

1. Introduction

Fusarium is a diverse genus, which includes pathogens of a multitude of economically important plant species, producing damping-off, root rot and vascular wilt diseases [1]. Plant cell wall is the first barrier that pathogens must break down to penetrate into the plants tissues [2], being one of its main components the pectin substances, a complex matrix of heteropolysaccharides composed mainly of galacturonic acid chains [3]. Pathogens produce a great number of pectin degrading enzymes among which polygalacturonases (PGs) are considered one of the most important [4].

Fusarium oxysporum f.sp. *radicis lycopersici* (FORL) produces in *in vitro* cultures several PGs isoforms and have been detected endo- and exopolygalacturonase activities [5] encoded by at least two endoPG genes (*pg1* and *pg2*) [6] and two exoPG genes (*pgx1* and *pgx2*) [7]. *Pgx1* showed similarity with *pgx4* [8] of *F. oxysporum* f.sp. *lycopersici*, *pgx1* of *Cochiobolus carbonum* [9] and *Aspergillus tubingensis* EXOPG coding gene [10] but although the gene has been described les is known about the characteristics of the protein. The other EXOPG produced by FORL, PGX2, has been previously purified [11] and the gene has been characterized [7].

Different roles during the course of interaction of fungi *in planta* or *in vitro* (on pectin) have been attributed to the endopolygalacturonases (ENDOPGs) and exopolygalacturonases (EXOPGs). The role of these enzymes may be determined not only by their pattern of expression during the saprophytic cycle and in the course of host infection, but also by their structural and physico-chemical features. Information about the differential expression patterns of PG coding genes is available for several pathogenic fungi [12] while less information is available on the physico-chemical features of PGs, particularly of EXOPGs [9,13-15]. This is largely due to the difficulties to obtain purified PGs from cultures where several isoforms are produced. Heterologous expression [16,17] of individual *pg* genes is a good approach to obtain and characterize PG enzymes. The *P. pastoris* systems presents several advantages being one of the most frequently used to produce eukaryotic proteins, furthermore, it is an organism generally regarded as safe (GRAS) and, in this case, there is no secretion of endogenous PGs.

In the present work, FORL *pgx1* gene was successfully expressed in *Pichia pastoris* and the enzyme produced was characterised. The physico-chemical characteristics of this enzyme were compared with those reported for PGX2 of the same strain of *F. oxysporum* f.sp. *radicis lycopersici* in native conditions.

2. Material and methods

2.1 Strains and plasmids

Yeast strain of *P. pastoris* GS115 and the expression vector pPICZαa were included in the kit EasySelect Pichia expression kit (Invitrogen). This vector contained the alcohol oxidase gene (*aox1*) promoter situated upstream of the α factor signal peptide, a multiple cloning site that allowed the introduction of a coding sequence and a His-tag. The *Escherichia coli* strain DH5α was used as host for plasmid pMOSBlue T-vector (Amersham Biosciences) containing the complete *pgx1* cDNA sequence.

2.2 Media composition and growth conditions

Bacterial clones were grown at 37ºC overnight in low-salt LB medium: 10 g/l peptone (DIFCO), 5 g/l NaCl (Panreac) and 5 g/l yeast extract (DIFCO, Detroit, USA) with Zeocin (25 µg/ml) (Invitrogen) and with 15 g/l agar (DIFCO) for the plates. *P. pastoris* was cultured on YPD (2 % peptone, 1 % yeast extract and 2 % glucose) and the transformants were selected on YPD plus Sorbitol 1M and Zeocin 100 µg/ml medium (YPDSZ).

2.3 DNA manipulation and cloning

The *pgx1* cDNA was isolated from plasmid pMOS Blue T-vector carrying the complete cDNA sequence of *pgx1* corresponding to the mature PGX1 protein by PCR using the oligonucleotides pgx1up 5´- TAGCG GAATTC GCC CAT GTT GAG GCT GCA –3 ´ carrying *EcoR* I restriction site and pgx1low 5´- CGTA GCGGCCGC ACC TCT ACC TTC AAT TCC ATT GGT TGT GTC CCT G-3´carrying *Not* I restriction site and the amino acid sequence recognised by a specific protease (Factor Xa) to allow the elimination of the His-tag. The amplification protocol was described previously [7] using a annealing temperature of 58ºC and 10 cycles. The PCR amplification product was cloned in pPICZαa vector, previously digested with *Not* I and *EcoR* I (Amersham Biosciences), according with the manufacturer's instructions. Ligation and transformation of *E. coli* were performed according to Sambrook [18]. The amplification fragment and restriction products were purified from agarose gels before digestion with endonucleases or ligation with the High pure PCR Product Purification (ROCHE) according to the manufacturer's instructions. The selection of transformants grown in selective medium was performed by PCR using the same oligonucleotides and PCR program indicated above, except for the number of cycles (30 cycles instead of 10). One positive clone was chosen and its plasmidic DNA was obtained and purified from an overnight culture using the Quiaprep Spin miniprep (Quiagen) according to manufacturer's instructions. The recombinant plasmid was named pPICZα-PGX1, and the correct sequence of the insert, containing the *pgx1* cDNA sequence fused with the signal peptide Factor α, was confirmed by automated sequencing using the DNA ABI-PRISM (Applied Biosystems) according to the manufacturer's instruction in the Genomic Unit of the Complutense University of Madrid.

2.4 Transformation of *P. pastoris*

pPICZαa and pPICZα-PGX1 were linearised with *Sac* I endonuclease. The plasmid preparations were concentrated up to 400 ng/µl by precipitation using the procedures described in Sambrook [18]. Transformation of *P. pastoris* GS115 was performed by electroporation using a BioRad Gene Pulser (BioRad) according to the EasySelect *Pichia* expression kit already mentioned. After transformation, colonies were grown on YPDSZ. Positive transformants were confirmed by direct PCR screening of cells using the same protocol as the one described above for bacterial clones.

2.5 Shake flask expression experiments

Five positive clones of *P. pastori*s and the negative control (pPICZαa) were used for expression experiments. They were grown in BMGY (0.4% yeast extract, 0.6% triptone, 50mM K_2PO_4 ph 6, 1.34% Yeast Nitrogen Base (DIFCO) with ammonium sulfate and without amino acids, 4 10-5 % Biotine, and 1% glycerol) liquid medium which was subsequently collected at different times (1, 2, 3 and 4 days) and resuspended in BMMY modified (0.4% yeast extract, 0.6% triptone, 50mM K_2PO_4 ph 6, 1.34% Yeast Nitrogen Base (DIFCO) with ammonium sulfate and without amino acids, 4 10-5 % Biotine, and 1% methanol) according to the protocol described in the instructions by Ease Select *Pichia* expression kit. Samples of each culture filtrate were taken and dialyzed against water for further analysis. The sample with highest PG activity was cultured in five flasks with 400 ml

of BMGY and transferred to 50 ml of BMMY modified according to the protocol to perform the characterisation assays.

2.6 Assays of PG activity

PG activity was measured by Somogy and Nelson method [19,20]. Polygalacturonic acid (0.1% w/v) (SIGMA) was used as substrate in 50 mM sodium acetate buffer in all assays except for the substrate affinity assays; in this case, polygalacturonic acid (SIGMA), digalacturonic acid (SIGMA) or trigalacturonic (SIGMA) were used as substrates in different concentrations. Determination of optimum pH was performed using different buffer solutions, prepared according to Geigy tables [21]. The pH stability assay included a pre- incubation of the samples for 30 minutes in the buffer solutions mentioned before followed by the assay of PG activity.

3. Results

The cDNA corresponding to the mature PGX1 enzyme of FORL was cloned in the expression vector pPICZαa and used to transform *P. pastoris* GS115. The cDNA was expressed and produced a functional PG protein, which was secreted into the extracellular culture. Five positive clones showing extracellular PG activity were obtained and the optimum culture conditions for PG expression were determined. Fig. 1 shows the results of PG activity produced by the five recombinant strains along the four days of culture. Absence of endogenous PG activity was confirmed from the analysis of the negative control (pPICZαa without insert).

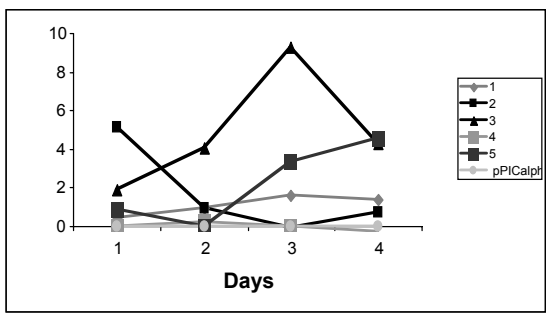

Fig 1. PG activity of five *P. pastoris* positive transformants at different days of culture. *P. pastoris* GS115 transformed with pPICZαa was used as negative control.

P. pastoris produced PGX1 could not be purified by immobilized metal-ion affinity chromatography what could indicate that the overall conformation of the protein was such as to inhibit the interaction between the His-tag and the immobilized Ni^{2+} ions.

However, taking into account that the protein was being produced in levels high enough as to be detectable using a specific activity assay we decide to pursue its kinetic and physico-chemical characterization. Recombinant strain number 3 was chosen for this analysis of the heterologously produced PGX1. Optimum pH and temperature, substrate affinity and enzyme stability in relation to pH and temperature were the features chosen to be studied.

The optimum pH of heterologous PGX1 was investigated in a pH range from 3.0 to 9.0. PGX1 activity had an optimum pH of 5.0, which sharply decreased to 50 % at pH lower than 4.0 or higher than 6.5 (Fig. 2a). The stability of the enzyme was also tested in the same range of pH and the results indicated a high stability (>80 % of the maximum PG activity) between 4.0 and 5.5 (Fig. 2b).

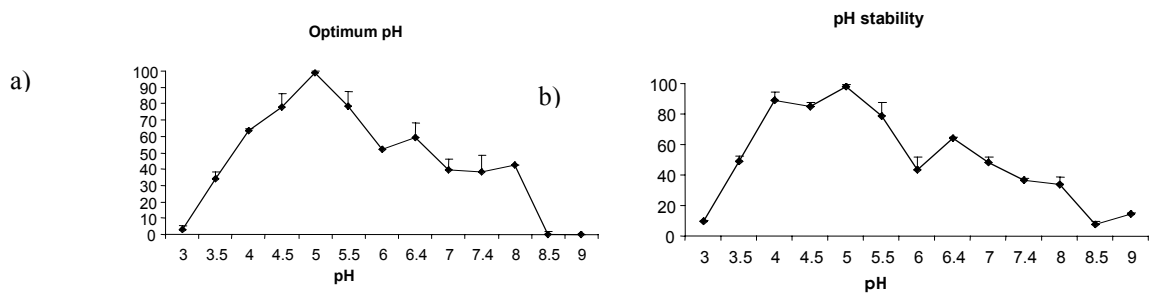

Fig. 2 Optimum pH (a) and pH stability (b) of heterologous PGX1.

The optimum temperature for heterologous PGX1 activity was analyzed in a range of temperature from 10 ºC to 70 ºC (Fig. 4a). The highest PGX1 activity was reached at 45 ºC and sharply decreased at temperatures higher than 50 ºC. Between 30 ºC and 45 ºC the enzyme retained about 60 % of the maximum PG activity. The stability of the enzyme was tested for periods of 5 minutes to 30 minutes at temperatures ranging from 10 ºC to 60 ºC. The results shown in Fig. 4b indicated that 100 % of the activity was retained when treated between 10-40 ºC during 30 minutes and decreased to 70 % at 50 ºC after 30 minutes treatment. The PG activity was severely affected after just 5 minutes at 60 ºC.

The kinetics of PGX1 for different substrates followed a Michaelis-Menten´s mode, showing different values of K_m and K_{cat} being the affinity of PGX1 for trigalacturonic and digalacturonic higher than for polygalacturonic acid. A summary showing the features of PGX1 described in this work and those reported for native PGX2 of *F. oxysporum* f.sp. *radicis lycopersici* are shown in Table 1.

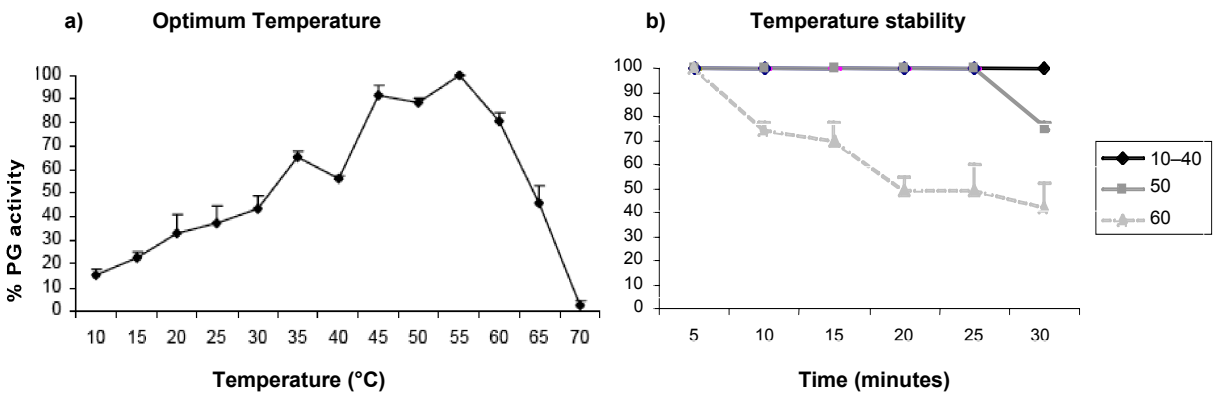

Fig. 3 Optimum temperatura (a) and temperature stability (b) of heterologous PGX1.

	OPTIMUM pH	OPTIMUM TEMPERATURE	SUBSTRATES AFFINITY (Km)		
			Poligalacturonic	Digalacturonic	Trigalacturonic
PGX1	5	55ºC	1.3 mg/ml	1.08 mg/ml	0.84 mg/ml
PGX2	5.5	60ºC	0.13 mg/ml	0.24 mg/ml	0.20 mg/ml

Table 1 Comparison between PGX1 and PGX2 biochemical characteristics.

4. Discussion

The expression of *pgx1* gene in *P. pastoris* produced a secreted and active form of PGX1. Previous attempts to express *pgx1* in *Saccharomyces cerevisiae* were unsuccessful in our hands, suggesting that *P. pastoris* might be a better alternative to obtain PG enzymes. The differences observed among the recombinant clones in the levels of PG activity could be attributed to differences in the pattern of integration of the expression cassette in the genome of *P. pastoris* which could affect the regulation of the gene. The presence of the His-tag in the recombinant protein did not help to purify the protein using immobilized metal-ion affinity chromatography (data not shown). These results could be due to a possible interaction between the protein and the His-tag preventing the interaction of the histidines with the Ni^{2+} ions of the affinity chromatography [22]. However, the PG activity present in culture filtrates of the clones obtained allowed the enzymatic characterisation of PGX1 and the comparison with the information available for the other EXOPG enzyme, PGX2, produced by *F. oxysporum* f.sp. *radicis lycopersici* [15].

Recombinant PGX1 showed an optimum pH of 5, similar to PGX2 [6,15] and was within the range of the fungal PGs described so far [13,23]. However, the pH stability of PGX1, showing 49% of activity at pH 3.5 and 10% at pH 9, was higher that the pH stability described for PGX2. The production of PGs with high stability in a broad range of pH could be very important in the process of colonisation, since it has been described that pH changes during the infection process [24].

The activity of PGX1 increased with the temperature up to a maximum at 55 ºC. The stability of this enzyme was high at temperatures ranging from 10 to 40 ºC, and lower than PGX2 [11], but in the range of other fungal PGs in native conditions [14].

PGX1 showed Michaelis-Menten´s kinetics with different values of K_m and K_{cat} for the different substrates tested. The affinity of PGX1 for trigalacturonic and digalacturonic acid agreed to its exopolygalacturonase

condition [25] being the highest affinity for trigalacturonic acid. The values of substrate affinity also showed differences between PGX1 and PGX2. In both cases, the values obtained for poligalacturonic and digalacturonic did not fell within those proposed for fungal PGs by Rombouts and Pilnik [26]. However, the variability is probably high, since extreme values of 0.09 mg/ml obtained for polygalacturonic acid in *Geotrichum lactis* [27] and 25 mg/ml for one EXOPG from *F. oxysporum* f.sp. *lycopersici* [28]have been reported. The existence of two EXOPGs with different substrate affinity has been also described in *A. niger* [29]. It should be taken into account that during the process of cell wall degradation, a complex mixture of potential PG substrates arises and varies along the time. The presence of PGs with different substrate affinity might increase the efficiency of the process and the symptoms produced.

Several studies failed to demonstrate an essential role of individual *pg* genes in pathogenesis, suggesting that the different PGs would be redundant and, therefore, the suppression of a particular *pg* gene could be compensated by the rest of the PG coding genes [8,9]. However, our results suggested that the redundancy of EXOPG enzymes is only apparent and the differences observed could be relevant not only during the process of infection but also during saprophytic growth providing the fungus with flexibility and higher efficiency which could be evolutionary advantageous. On the other hand, the production of PGs with different physico-chemical features is of great interest in food industries which make use of PGs in a variety of processes with different requirements and conditions. The possibility to express individual *pg* genes to produce a variety of functional PGs will offer a good approach to exploit the high variability observed in fungal PGs.

Acknowledgments This work has been supported by MCyT (BMC2000-1458) (Spain) and A. de las Heras by a UCM pre-doctoral fellowship (Spain).

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In silico and *in vitro* analysis of promoter regions of two exopolygalacturonase coding genes of *Fusarium oxysporum* f.sp. *radicis lycopersici* and regulation in *Saccharomyces cerevisiae*

A. de las Heras[1], M.T. González Jaén[1], B. Patiño[2], F.J. Ruíz-Dueñas[3], A. Villalvilla[1] and C. Vázquez[2,*]

[1] Departamento de Genética and [2]Departamento de Microbiología III, Universidad Complutense de Madrid (UCM), José Antonio Nováis 2, 28040-Madrid, Spain.
[3] Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CSIC), Ramiro de Maeztu 9, 28040-Madrid

[*]Corresponding author: e-mail: covi@bio.ucm.es, Phone: +34 913 944 442

Fusarium oxysporum f.sp. *radicis lycopersici* (FORL) causes an important disease in tomato characterized by cell wall degradation. The characterization of cell wall-degrading polygalacturonase enzymes and their regulation is crucial to gain knowledge on the role of these enzymes in pathogenesis. The objectives of this work were to perform an *in silico* and *in vitro* analyses of the upstream regions of two exopolygalacturonase (EXOPG) coding genes of FORL and to develop an easy and reliable system to perform the functional analysis of those promoters. The analysis of the upstream regions of *pgx1* and *pgx2* genes revealed differences in the distribution of regulatory motifs. These upstream regions were fused with the ß-galactosidase reporter gene in a *Saccharomyces cerevisiae* vector. The induction of the reporter gene was analyzed in response to different carbon sources and the results compared to the induction pattern of *pgx1* and *pgx2* genes in *in vitro* cultures of FORL.

Keywords exopolygalacturonases; Fusarium oxysporum f.sp. radicis lycopersici; Saccharomyces cerevisiae; promoters; regulation

1. Introduction

Fusarium oxysporum Schlecht. f.sp. *radicis lycopersici* Jarvis and Shoemaker (FORL) [1] is responsible for foot, crown and root rot disease in susceptible tomato plants. These symptoms involve cell wall degradation and extensive tissue maceration. Pectinases, especially polygalacturonases (PGs), hydrolyze pectin located in the middle lamella of host cell wall, thus facilitating cell breakdown and colonization of host tissue [2]. Fungi, bacteria and a few *Saccharomyces* strains produce a number of PGs resulting from several PG encoding genes [3-6]. Few reports on targeted disruption of fungal PG coding genes indicate a critical role of individual *pg* genes in pathogenesis [7]. However, the existence of complex arrays of PG enzymes showing different structural features, exo- or endo modes of action (ENDOPG and EXOPG, respectively), diverse optimum pH or substrate preferences and some differential regulation in response to diverse conditions [8-10] suggest that their impact on colonization could be a consequence of their effective coordination. The study of the regulation of the PG synthesis has mainly been performed in *in vitro* cultures, where conditions can be controlled and factors individually tested. Four PG coding genes have been identified in FORL strain r6. Two of them encoded ENDOPGs (*pg1,* GenBank accession AY485190 and *pg2*, GenBank access. AF078156) and two encoded EXOPGs (*pgx1*, GenBank acces. AF395107 and *pgx2*, GenBank acces. AF136444).

The expression analysis of both EXOPGs coding genes indicated that they were transcriptionally induced in response to carbon sources such as apple pectin and galacturonic acid, and subjected to catabolite repression [8, 9]. Recent reports indicate that some PGs are regulated by pH and developmental clues [11-13], this fact suggests that the expression of the PG coding genes is under the control of a complex of regulatory pathways.
The determination of putative regulatory elements located in the promoter regions of PG coding genes by using an *in silico* analysis, is necessary to identify new regulators that can be controlling the expression of the PG genes. The subsequent functional analysis in a simple and efficient expression system is a straightforward approach to unravel the conditions in which the expression of those genes is affected. The development of a reliable heterologous system to perform this functional analysis of promoters would be extremely valuable since *Fusarium* has low transformation efficiency and expression of integrated constructs may be affected by copy number and position. In this study, we report the expression analysis of the promoter regions corresponding to the two EXOPG coding genes of FORL r6 with respect to the carbon source in *Saccharomyces.* The present work confirmed the usefulness of the *Saccharomyces* model to analyze common regulatory pathways in fungus and yeast using a simple and rapid method.

2. Experimental procedures

2.1 Strains and culture conditions

The FORL r6 strain was initially obtained from infected tomato plants from the Spanish Mediterranean coast by Dr. J. Tello (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain) and was maintained as stock cultures on potato-dextrose agar (PDA) slants at 4ºC and as micro conidial suspensions in 15% glycerol at -80ºC. Clones were amplified in the *Escherichia coli* DH5α strain. *S. cerevisiae* ATCC28583 a derivative of FL100 pectinolytic activity-deficient control strain was kindly provided by Dr. Belarbi (Université de Reims, France). *S. cerevisiae* was cultivated in liquid medium YPG (1% yeast extract, 1%, Bactotriptone and 1% glucose). The transformants were selected in minimal medium: YNB without amino acids (Difco, Detroit, USA) supplemented with 0.002% tryptophan and 2% glucose (SIGMA). The *S. cerevisiae* strains carrying different plasmids were grown in liquid medium YNB with amino acids supplemented with different carbon sources (SIGMA): 0.5 and 1.0% glucose (Glu), 0.5 and 1.0% apple pectin (AP), 0.5 and 1.0% galacturonic acid (GalA), 0.5 and 1%, polygalacturonic acid (PGal), 0.5% Glu + 0.5 and 1.0% GalA, 0.5% Glu + 0.5 and 1.0% PGal and 0.5% Glu +0.5 and 1.0% AP.

2.2 Construction of plasmids

Promoter regions of the *pgx1* and *pgx2* genes of FORL were obtained from the genomic clones of *pgx1* (GenBank acces. AF395107) and *pgx2* (GenBank acces. AF136444). The 631 bp upstream fragment of *pgx1* and the 668 bp upstream fragment of *pgx2* were generated by PCR amplification using the Pwo polymerase(ROCHE) according to the manufacturers instructions. The oligonucleotides used as primers were: pgx1/1F (5´GCATGGATCCAATTCTCTCATGCAATCAAGTT3´) and pgx1/2R (5´ACTATCTAGAGAACAACATGTTGATTCTCGA3´) for *pgx1* gene and pgx2/1F (5´GCATGGATCCTCTCGCATGATGTCCGTCC3´) and pgx2/2R (5´ACTATCTAGAGACCTTCATTGTGAAAAGATT3´) for *pgx2* gene.

The plasmid YEp357, kindly provided by Dr. Daignan-Fornier (University of Bordeaux II, France), was used for the constructions with promoter regions of EXOPG genes of FORL strain r6 as it is described elsewhere [8]. The plasmid YEppgx1 carried the 631 bp *pgx1* promoter upstream region of the *pgx1* gene, and the plasmid YEppgx2 carried the 668 bp upstream region of the *pgx2* gene. The sequences of the inserts in YEppgx1 and YEppgx2 were verified by sequencing using an ABI PRISM DNA Sequencer (Applied Biosystems) following the manufacturer's instructions in the Genomic Unit of the University Complutense of Madrid (Madrid, Spain) The *S. cerevisiae* ATCC28583 strain was transformed with 1 µg of YEp357, YEppgx1 and Yeppgx2 plasmids according to the method described by Eble [14].

2.3 β- Galactosidase assay

β-galactosidase activities were measured on whole cells by the method of Kippert [15]. The activities are given as the ratio between the arbitrary units (AU), defined by Kippert [15], and the value of biomass of the culture estimated as OD600nm. Values and averages were obtained from at least triplicate experiments.

2.4 Analysis of the promoter regions' sequences

TRANSFAC software available at www.transfac.gbf.de [16] was used to detect putative regulatory motifs in the upstream regions of the *pgx1* and *pgx2* genomic clones.

3. Results

3.1 Analysis of the upstream region of *pgx1* and *pgx2* genes

Two EXOPG coding genes from FORL had been obtained by the screening of a genomic library. These genes were present as single copy in the genome of the fungus and their low similarity (28.3 %) when the amino acid sequences were compared suggested that they were distinct genes. The genomic clones containing *pgx1* and *pgx2* genes included a non-coding upstream region of 631 bp and 668 bp, respectively, which showed low similarity (22.0%). Nevertheless, both upstream regions basically shared the same motifs (Fig. 1). Five CREA-binding motifs, related to carbon catabolism [17], were detected at different positions. GATA motifs, related to nitrogen metabolism and iron regulation [18], were found in the promoter region of both genes. Two regulatory motifs related to stress were also detected: the "Heat shock element" [19], present in both promoters, and the "STRE motif" [20] present only in the promoter region of *pgx1*. The regulatory motifs related with

developmental control, StuAP of *Aspergillus nidulans* [21] and Aba /Tec1p of yeast [22, 13], were only present in the promoter region of *pgx2*.

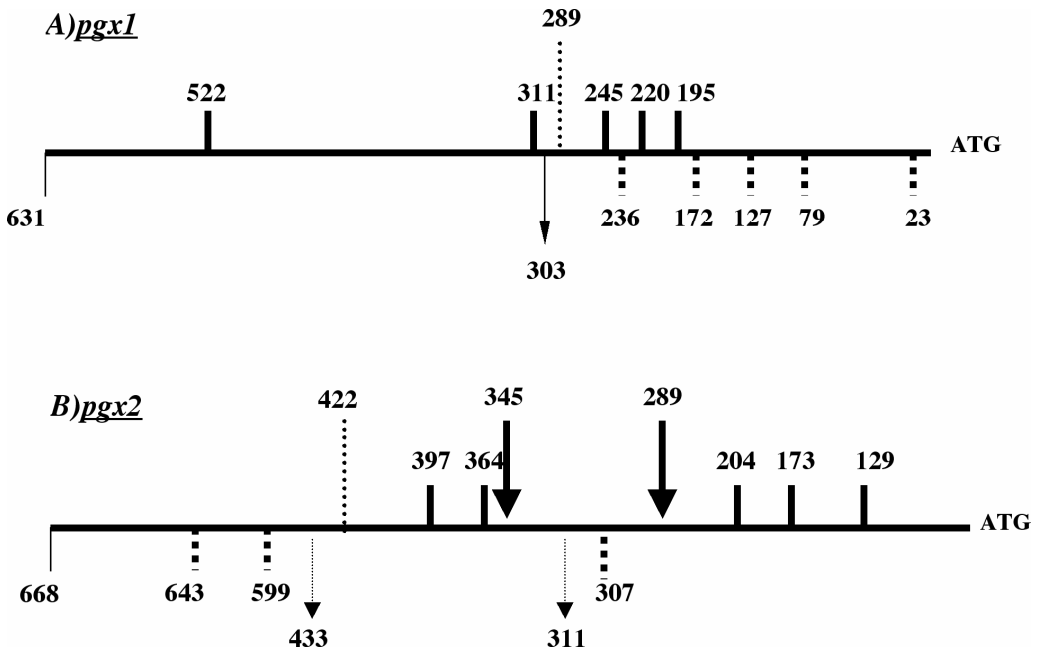

Fig. 1 Location of putative regulatory motifs in the *pgx1* (A) and *pgx2* (B) upstream regions. CREA motifs (5´-G/CYGGGG-3´) are indicated by continuous black lines, GATA motifs (5´- TATCTA-3´) by discontinuous black lines, Heat Shock (5´-NGAAN-3´) by dotted lines, STRE (5´- AGGGGG-3´) by thin continuous black arrows, StupAP (5´-A/TCGCGT/ANA/C-3´) by discontinuous arrow and AbaA (5´-CATTCY-3´) by bold arrows. The numbers indicate the position of the motifs upstream the *pgx1* and *pgx2* ATG codons.

3.2 Expression of the promoter regions of *pgx1* and *pgx2* of FORL in *S. cerevisiae*

The ability of the upstream regions of *pgx1* and *pgx2* to induce the reporter gene, β-galactosidase, has been tested in *S. cerevisiae* in response to several carbon sources. The chimeric plasmids Yeppgx1, Yeppgx2, which contained the 631 bp and 668 bp long upstream regions of *pgx1* and *pgx2*, respectively, and the negative control, Yep357 were used to transform *S. cerevisiae* ATCC28583 strain. Plasmid-containing cells were grown on YNB medium supplemented with the different carbon sources and the β-galactosidase activities determined at different times during culture. The results are shown in Fig. 2A and 2B.

The upstream regions of *pgx1* and *pgx2* [8] were able to induce β-galactosidase expression in *S. cerevisiae.* Galacturonic acid, polygalacturonic acid, and apple pectin induced β -galactosidase expression while glucose neither alone nor in combination with the other carbon sources was able to drive β-galactosidase expression.

The expression pattern of both promoters showed differences in level and time of induction. The *pgx2* promoter showed higher levels of β- galactosidase induction and at earlier times than the *pgx1* promoter. Differences regarding the carbon source or the concentration used were not detected except for apple pectin, which showed slightly less induction than polygalacturonic acid or galacturonic acid.

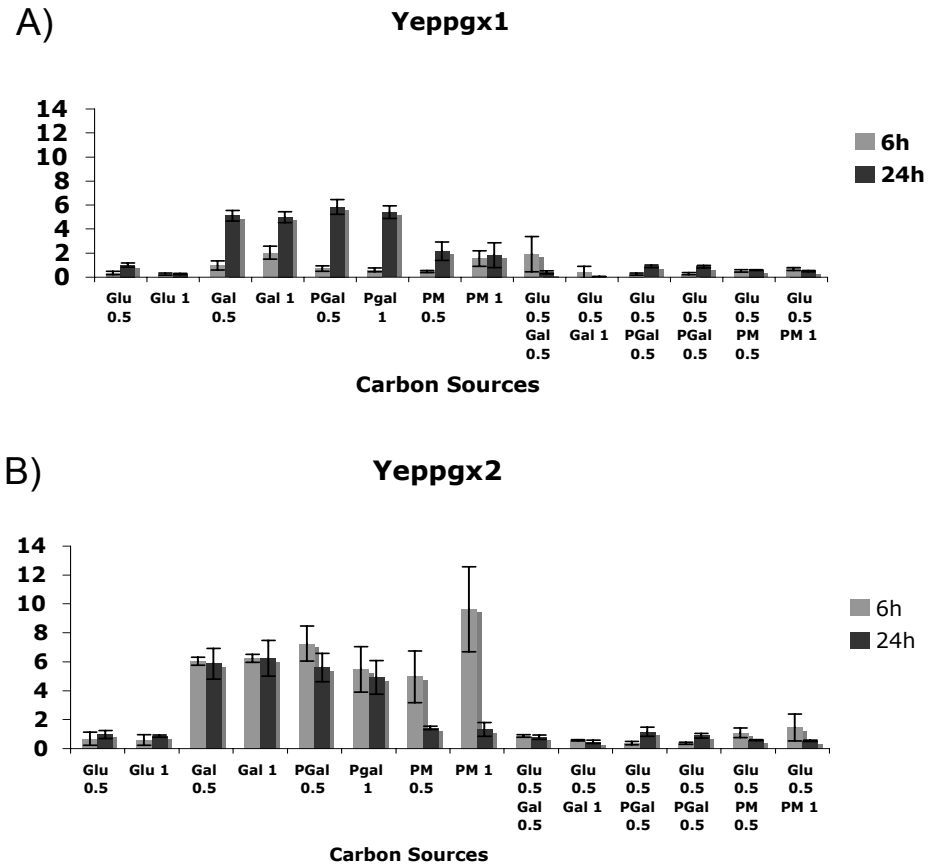

Fig. 2 ß-galactosidase activity of *in vitro* cultures of *S. cerevisiae* carrying the plasmids Yeppgx1 (A) and Yeppgx2 (B) supplemented with different carbon sources glucose (Glu), galacturonic acid (GalA), polygalacturonic acid (PGal) and apple pectin (AP). The cultures were supplemented with 0.5% (0.5) and 1% (1) of the specified carbon sources.

4. Discussion

We have performed an analysis *in silico* of the promoter regions of the EXOPG coding genes of FORL in order to determinate which factors, other than those related with carbon sources, can be affecting their expression (Fig. 1): Putative CREA regulatory motifs related with catabolite repression [17] have been identified in the promoter regions of the EXOPG coding genes of FORL. The presence of these CREA motifs fits in model of repression by glucose reported for other PG enzymes [12,9,23]. GATA motifs related with the metabolism of nitrogen, stress related motifs (Heat shock element and STRE motifs) and regulatory motifs related with developmental control (StuAP and Aba/Tec1p) were differentially present in both promoters suggesting a possible role of those factors in the regulation of EXOPG coding genes. The co-regulation of an ENDOPG and filamentation and invasive growth in *S. cerevisiae,* induced by nitrogen starvation and controlled by the MAPK pathway [24], probably corresponds to the situation observed in yeasts [12] and in pathogenic fungi [25,26]. These observations suggest a complex regulation of PGs which would primarily respond to carbon sources but also to signals from the environment or the host in order to coordinate successfully both nutrition and host infection. On the other hand, they suggest the conservation of basic regulatory mechanisms in yeast and filamentous fungi. The results of our study also support this idea.

The expression of two EXOPG genes of FORL r6 in relation to carbon source has been analyzed using the promoter regions of both genes fused to a reporter gene in *S. cerevisiae.* The expression analysis of these genes in the heterologous system was performed in response to carbon sources in order to compare the results with the data obtained in FORL cultures [8]. The results indicated the ability of this system to induce both promoters in response to the carbon sources used. Both promoter regions were able to induce the expression of the reporter gene in the presence of apple pectin, polygalacturonic acid, and galacturonic acid, and both were repressed by glucose (Fig. 2). The induction of both promoters by apple pectin, polygalacturonic acid and galacturonic acid, and the repression by glucose in *S. cerevisiae* was similar to the pattern observed for those genes in *in vitro* cultures of FORL by Northern blot analysis [8]. In this study, *pgx1* and *pgx2* genes were transcriptionally induced by pectin, polygalacturonic acid and galacturonic acid and repressed by glucose. This regulation pattern

basically fit in a model of catabolite repression, also reported for other PG enzymes from diverse fungi and yeasts [12,9,23], suggesting a common regulatory mechanism for these enzymes, which could be mediated by CREA regulatory motifs [17]. This fact is also supported by the high percentage of identity (79%) between the *F. graminearum* CREA protein (Genbank EAA77764) and the *S. cerevisiae* MIG1 transcription factor, the main component of the glucose repression pathway [27]. Several CREA motifs have been detected in both *pgx1* and *pgx2* promoters, although selective deletions of the several CREA motifs present in both promoters should be tested in order to determine which of them are actually functional. This analysis will be facilitated by the use of the system described in this work to study promoter expression in *S. cerevisiae*. Polygalacturonic acid and galacturonic acid showed a similar ability to induce the expression of the reporter gene and higher ability than apple pectin (Fig. 2). However, apple pectin was a good inducer of the transcription of *pgx1* and *pgx2* genes in *in vitro* cultures of FORL r6 [8]. Low levels of other pectinases secreted by FORL in these cultures, including ENDOPGs, could generate additional inducers resulting in an increase of *pgx* expression. The expression of the *pgx2* promoter was earlier detected and reached higher levels than that of the *pgx1* promoter, similarly to the expression pattern of *pgx1* and *pgx2* observed in *in vitro* cultures of FORL [8], indicating that *Saccharomyces* was able to recognize and interpretate the information contained in both promoters.

These results support the hypothesis of differential contribution of PG genes mediated by differences in regulation and, in the case of FORL r6, the important contribution of *pgx2* to the PG activity produced by this fungus. The different array of regulatory motifs in both promoters could be responsible from such differences and suggests, on the other hand, differences in the signals, which they are probably able to recognize. This could lead to a certain specialization of these enzymes in the processes of infection and symptoms development. The regulation of many fungal genes could benefit from the knowledge of yeast regulatory mechanisms and the tools developed in yeast facilitating a direct and rapid approach to evaluate putative regulatory motifs present in their promoters.

Acknowledgments This work has been supported by MCyT (BMC2000-1458) (Spain) and A. de las Heras by a UCM pre-doctoral fellowship (Spain).

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Modification by transposition in the bacterial production of poly-β-hydroxybutyrate (PHB) in *Azospirillum brasilense*

Hector Patricio Ibarra Campeche, Reyna Álvarez Martínez, Lucía Soto Urzua, and L. J. Martínez Morales*

Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla. Edif. 76 Complejo de Ciencias, C. P. 72570. tel (52) 222 244 45 18. Puebla, Pue. México.

*Corresponding author: jlmartin@siu.buap.mx

Poly-hydroxy-butyrate (PHB) is one epoxy plastic secondary of petroleum that can support to resolve problems of environmental pollution by yours mould, resistance, and biodegradable characteristics. PHB has been identified over of twenty bacterial genus inside of the which is find *Azospirillum*. In bacterial, your function has been suggested to provide reserves of carbon and reductant that are important in sustaining respiratory activities to protect nitrogenase from damage by O_2 and in extending nitrogen fixation. The PHB accumulates reserves in conditions of unbalance of nutrients. By yours applications is very important to increase our understanding of the role of PHB in the species bacterial *Azospirillum*, by generation of the bacteria hyperproducer of PHB, was obtained by genetic handing and give application in the plastics industry. In this work mini-Tn5-Km mutagenesis was performed in the wild-type strain *Azospirillum brasilense* the mutants strains obtained were selected by staining with Sudan black and that transconjugants considerably interesting were realized analysis of the influence of the ammonium chloride (NH_4Cl) over the production of PHB. Two mutants named 2 and 5, were selected, quantified PHB production, showing the better production that wild type, consistent with data of dried weigth and pH variation. The best K_La assayed was 8.2 h^{-1} and the better concentration of the NH_4Cl was 0.15 g/L for the wild-type strain and 0.5 g/L for the mutants 2 and 5. The PHB production was quickly in the mutants more than the wild-type strain, by space of 20 h. Is look at the producing PHB when NH_4Cl was consumed.
Has been cloned in the pBluescript plasmid, one fragment of 2.0 kb DNA containing 0.8 kb of DNA of *A. brasilense* obtained by mutation generated by the insertion of mini-Tn5-Km. The analysis "*in silico*" feature the similarity of the secuence with the involved genes.

Keywords poly-ß-hydroxybutyrate (PHB), poly-ß-hydroxyalcanoates (PHAs), *Azospirillum.*

Introduction

The poly-ß-hydroxyalcanoates (PHAs) are polyester polymers of the several hydroxyalcanoates they are synthesized by several microbial cells as material of reserve and energy. PHAs accumulate in the citosol of microbial cells under limiting nutrient essential N/P in your ambient and underbalanced growth conditions C/N (Wang *et al*. 1998). The PHAs are biodegradable polymers and have similar properties to different thermalplastic and synthetic elastomero, which one propylene, synthetic rubber (Anderson *et al*. 1998). PHB is a member of the class of the poly (3-hydroxyalcanoates) PHAs (Marchessault *et al*. 1990). Nitrogen fixing rhizobacterium *Azospirillum brasilense* lives in close association with plant roots, where it exerts benefical effects on plant growth and yields of many crops of agronomic importance (Okon *et al*. 1994; Okon *et al*. 1997). PHB is find as intracellular body generally have a size between 200-500 nm (Anderson *et al*. 1998). In *A. brasilense* strain it was observed those has main contain of PHB have major resistance stress conditions, such inanition or radiation (Matin *et al*. 1979). *A. brasilense* Sp7 strain it was identified one genetic region involved in biosynthetic pathway, this region include three genes: *phbC* (PHB synthase), *phbA*(ß-ketothiolase) and *phbB* (acetocetyl coenzyme A reductase). The reason which one accumulate PHB in which oxygen and nitrogen sources are limiting continuous culture. *Azospirillum sp*. in continuous culture store up PHB in lactate limitation (Matin *et al*. 1979) and with NH_4^+ store up as far as 12% of your biomass (DOT= 0.007 atm and D = 0.14 h^{-1}). *A.brasilense* store up more 80% del PHB dry cell weight.

Production biodegradable plastics industrial of PHB is comparatively small and it has obtain fermentation bacterial process, has been a great impediment the commercial production to great scale by the high cost. By the before in this study we report the influence of NH_4Cl in the production of PHB by *A. brasilense*.

Objective

Determinate of the production of PHB to differents concentrations of the ammonium chloride in *A. brasilense*.

Material and Methods

Strains. *A. brasilense* UAP-154 wild type strain, mutants obtained by mini-Tn5-Km mutagenesis.

PHB production

Determinations were to carry out 15, 30, 45 and 60% of the fill volume with minimal medium supplemented with ammonium chloride to a final concentration of 0.0; 0.15; 0.5 and 1.0 g/L, were grown, and incubated on a rotary shaker at 160 rpm for 85 h at 32 ^{o}C. The aliquots of precultures were take out minimal medium without NH_4Cl.

Dry cell weigth and PHB production vs time.

Was determinated to K_{La} of 8.2 h^{-1} in minimal medium with 0.15 g/L of NH_4Cl with preculture same media. Was determinated to several times differents (to 85 h).

Cuantitative determination PHB

The extraction PHB with organics solvents and H_2SO_4 carried out formation crotonic acid, taked one mililiter samples culture, were centrifugated, the pellet was additionated one milliliter sodium hipochloride (clorhidre active 6%), was incubated in agitation to 37 ^{o}C two hours, was centrifugated, the pellet was washed with one milliliter of water, and then with one milliliter of acetone an the finish with one milliliter of ethyl alcohol (was dried).

The pellet was followed by 1 ml hot chloroform and was vigorously vortexing, take 0.1 ml and take off evaporation the chloroform, its add 5 ml concentrate H_2SO_4 put the top on, heat ebullition at 100 ^{o}C by 15 min. The PHB was change in crotonic acid, was detected by spectrophotometer at DO_{235} maximum absorption. Was realized standard curve DO_{235} vs mg of PHB (converted to crotonic acid).

Kinetic of consumption NH_4Cl

Was determinated by the ammoniacal nitrogen method. Total nitrogen as ammonium ion or equilibrium was considered ammoniacal nitrogen. Take off 500 ml diluted sample with 500 ml distilled water. The sample was declorhidre with 1.6 ml solution sodium tiosulphate, neutralized at pH 7.0, used H_2SO_4 or NaOH 0.1 N, added 25 ml borate buffer pH 9.5 with NaOH 1 N, was transferred the sample to Kjendahl, added 5 bead glass and was distilled. The distilled was reciped in 50 ml adsorbent and indicator solution of boric acid. The presence of ammoniacal nitrogen gave green blue coloration. If will be ammoniacal nitrogen was tritrated with 0.2 N H_2SO_4 standard solution to change the green blue to purple-violet coloration.

Kinetics of consumption Succinic acid

Was performed by organic material no volatile method. Consisted in oxidation of sample with excess of dichromate in sulphuric acid. Determination of dichromate initial by reduction with sodium sulphite and the reduction by the sample, the different is proportional to content of organic material no volatile, centrifugated 1 ml of culture, the supernatant was take of one aliquot of 0.4 ml was contained between 100-500 µg solids organics, was added 1 ml oxidant agent, mixing and ebullition at 95 ^{o}C during 45 min and quickly cool. Was realized two blanks one with 0.4 ml of water in place to sample and other with 10 mg of Na_2SO_3, they were reading in the spectrophotometer to DO_{440}, the blank reduce was take with cero absorbance and the others was reading vs these.

Hibridization assay

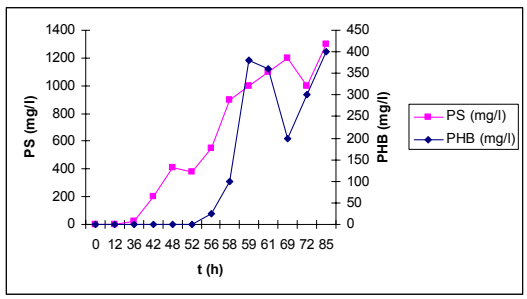
Routine DNA manipulations were essentially as in Maniatis et al. *Azospirillum* genomic wild type strain DNA was isolated with a Promega genomic DNA kit, restriction, ligations, transphormation and cloning were realized with Invitrogen kits.

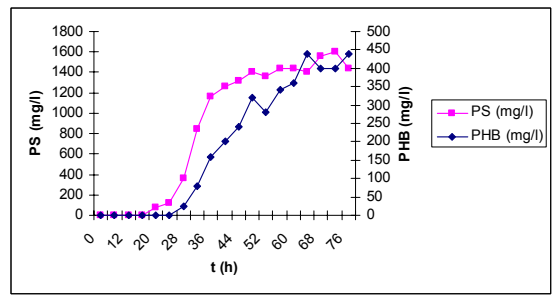
Results

In the wild type strain, the determination of pH in the culture showed one change at the 58 h, reaching pH 8.7, the succinic acid (as sole source carbon) reduce until 2 g/L concentration. The dry cell weigth increase to from

46 h reaching 1200 mg/L concentration, whereas PHB was detected up to 56 h having one maximum of 400 mg/L concentration, the assay with NH_4Cl 150 mg/L concentration falled to zero in only 40 h coinciding with PHB biosynthesis polymer.

For the case mutant assayed, pH and succinic acid behavior was very similar value but very different in time, the change pH at 40 h but not at 58 h as the wild type strain. For the dry cell weigth the change occurred at 24 h and PHB at 36 h with similar values. The NH_4Cl falled at zero at 30 h and is at 36 h the detection PHB.





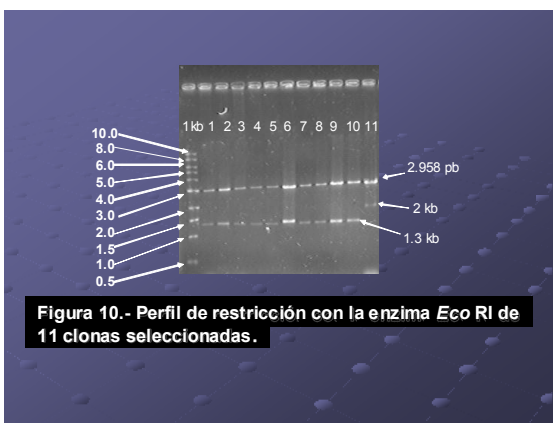


Figura 10.- Perfil de restricción con la enzima *Eco* RI de 11 clonas seleccionadas.

Has been cloned in the pBluescrip plasmid. One fragment of 2.0 kb DNA containing 0.8 kb of DNA of Azospirillum brasilense obtained by mutation generated by the insertion of a km resistance cassette (1.2 kb).

Discussion

Between the mutant and the wild type strains, the dates show one behavior similar values, so much succinic acid, pH, consumption NH_4Cl and dry cell weigth, however of biosynthesis of polymer time was very different, approximately 20 h before in the mutant, very important in the fermentation industrial, the costs reduction maintenance the big fermentors and as a consequence reduce the price of the product. Concluding the mutation is in the regulator gene, but is necessary the widely the characterization.

Acknowledgments: This work was supported by grant from VIEP-BUAP. Ref. 30/G/NAT/05.

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Preparation and characterization of Taxol loaded magnetic polymeric nanospheres

M.Koneracká[*,1], **V.Závišová**[1], **M.Múčková**[2], **A.Juríková**[1], **N.Tomašovičová**[1], **G.Lancz**[1], **P.Kopčanský**[1], **M.Timko**[1] and **M.Fabián**[3]

[1] Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 040 01 Košice, Slovakia
[2] Hameln rds a.s., Horná 36, Modra, Slovakia
[3] Institute of Geotechnics, Slovak Academy of Sciences, Watsonova 45, 040 01 Košice, Slovakia

[*]Corresponding author: e-mail: konerack@saske.sk, Phone: +421 55 792 22 36

In this study, we have prepared PLGA (poly(D,L-lactic-co-glycolic) acid) nanospheres (NPs) loaded with magnetic fluid (MFPEG) as a magnetic carrier and anticancer drug Taxol by the nanoprecipitation method. The PLGA was utilized as a capsulation material due to its biodegradability and biocompatibility. Taxol – an important anticancer drug was chosen for its significant role against a wide range of tumours. The morphology and particle size distributions of the prepared NPs were investigated using scanning electron microscope (SEM) and PCCS technique. The results confirmed the spherical shape of the prepared NPs with size ~ 200 – 250 nm in diameter. The differential scanning calorimetry (DSC) and infrared spectroscopy (FTIR) measurements confirmed the incorporation of Taxol into the magnetic PLGA NPs. Then the effect of ionic strength on the colloidal stability of PLGA, magnetite, Taxol, and composite nanospheres are shown. Finally, the results of toxicity of prepared samples are presented.

Keywords drug targeting, magnetic fluids, anticancer drug, nanosphere

1. Introduction

Drug targeting is defined as selective drug delivery to specific physiological sites, organs, tissues, or cells where a drug's pharmacological activities are required. In principle, a drug distributes in the whole body when it is injected in the blood, and the drug that is distributed to sites other than the therapeutic sites may cause toxic side effects. By increasing delivery to the therapeutic sites and by reducing delivery to the unwanted sites, an improved therapeutic index can be obtained with enhanced and reduced drug action at the therapeutic and the unwanted sites, respectively. Magnetically controlled drug targeting is one of the various possibilities of drug targeting. This technique is based on binding a selected anticancer drug with magnetic fluids into a pharmacologically stable formulation. The drug can be dissolved, entrapped, encapsulated or attached to nanoparticles and depending upon the method of preparation, nanoparticles, nanospheres (NPs) or nanocapsules can be obtained.

Magnetic–polymeric NPs, made from organic and inorganic components, have unique characteristics due to the specific properties of the blend. The constituents of a magnetic–polymeric NPs play different roles: the polymeric matrix acts as a shell, reservoir, and vehicle for the active component, whereas magnetite is the component which makes targeting possible by external magnetic field manipulation. The polymer matrix of the NPs must fulfil several requirements such as biocompatibility, biodegradability, mechanical strength, and ease of processing. The best known class of biodegradable materials for controlled release are the poly(lactide-*co*-glycolide)s (PLGAs). The main advantage of these degradable polymers is that they are broken down into biologically acceptable molecules that are metabolized and removed from the body via normal metabolic pathways. Paclitaxel (Taxol®), an important anticancer drug, has been chosen for encapsulation to the polymer for its significant role against a wide range of tumours (breast cancer, ovarian carcinoma, lung cancer, head and neck carcinomas) [1]. Taxol is a natural diterpenoid alkaloid, firstly isolated from the bark of the yew *Taxus brevifolia* [2]. It is not affordable from the nature. The other limitation of Taxol applications is its high insolubility in water and most pharmaceutical solvents. Adjuvants such as Cremophor EL have to be used in its current clinical administration, which, by itself, causes serious side effect - hypersensitivity reaction. In order to eliminate the side effects of the adjuvant and improve the therapeutic efficacy of the drug, alternative dosage forms have been suggested, including liposomes, microspheres and polymeric nanoparticles. Other important advantages associated with the use of nanoparticles include the simplicity of their preparation with well-defined biodegradable polymers and their high stability in biological fluids and during storage.

In this work we describe a technique for the preparation of nanospheres, consisting of a magnetic fluid MFPEG and PLGA as a matrix, to develop the stable magnetic nanospheres for Taxol delivery. Taxol was efficiently encapsulated by modified nanoprecipitation technique. The FTIR and DSC measurements were used

to confirm incorporation of magnetic particles and drug in the PLGA polymer. These prepared nanospheres were also characterized in terms of morphology, size and colloidal stability under conditions of different concentrations of aqueous solutions of NaCl. Finally, in vivo experiments of toxicity of prepared samples are presented.

2. Materials and methods

Taxol was obtained from Indena company. Poly(D,L-lactide-*co*-glycolide) (PLGA) with D,L-lactide to glycolide ratio of 85:15 and molecular weight of 50 000–75000 $g.mol^{-1}$, Pluronic F68 were purchased from SIGMA company and Poly(ethylene glycol) (PEG) with M_W =1000 $g.mol^{-1}$ was supplied by Merck.

The co-precipitation method of ferric and ferrous salts in an alkali aqueous medium was used to prepare magnetite particles [3]. TEM showed nearly spherical shape of freshly prepared magnetite particles with average diameter D_V = 12 nm and standard deviation $\sigma = 0.1$ nm. The magnetic properties of the particles were characterized by SQUID magnetometer at room temperature and a superparamagnetic behaviour was confirmed (data not shown). Superparamagnetism in drug delivery is necessary because once the external magnetic field is removed, magnetization disappears (negligible remanence and coercivity) and thus agglomeration (and the possible embolization of capillary vessels) is avoided. To prepare stable colloidal suspension of magnetic particles, sodium oleate ($C_{17}H_{33}COONa$) as a first surfactant was used to prevent their agglomeration. The key parameters of the behaviour of magnetic particles are related to the nature of their surface. The chemical composition of the surface is especially important to avoid the action of the reticuloendothelial system (RES), which is a part of the immune system, in order to increase the half-life in the blood stream. Coating the magnetic particles with a neutral and hydrophilic compound - poly(ethylene glycol) (PEG), the circulatory half-life increases from minutes to hours or days. Thus, PEG as a second surfactant was added to the system magnetite – sodium oleate. The modified nanoprecipitation method was used to entrap magnetic fluid (MFPEG) and anticancer drug Taxol into polymer nanospheres [4]. Briefly, 100 mg of PLGA and 5 mg of Taxol were dissolved in 10 ml acetone to prepare the organic phase. Next, the aqueous suspension was prepared by mixing 25.3 mg Pluronic F68 as a stabilizing agent, and 0.5 ml MFPEG (0.1 g Fe_3O_4/ml). Then, the organic phase was added drop wise into the aqueous suspension and stirred vigorously for several hours to allow complete evaporation of the organic solvent at room temperature. A turbid nanosphere suspension was formed.

Scanning electron microscopy (SEM, JEOL 7000F microscope) was used to observe the morphology and microstructure of Taxol loaded magnetic PLGA composite nanospheres. The colloidal dispersions were first diluted (typically 0.1 % solid content), then one droplet was deposited onto an aluminium grid and dried under vacuum. After sputtering with carbon, the samples were observed. The particle size distribution of prepared samples have been also measured by PCCS technique (photon cross correlation spectroscopy, Nanophox, Sympatec GmbH, Germany). The samples have been placed in the temperature controlled sample holder at least five minutes before starting the measurement. From each sample three cross correlation functions (CFF) were measured over periods of 600 seconds. The laser intensity and cuvette position was adjusted to ensure an average count – rate at the detectors of 300 kcps. In order to characterize the thermal behaviour of the prepared NPs, DSC was performed using PERKIN ELMER DSC 7 calorimeter. From the DSC studies of NPs the physical state of drug in the NPs can be investigated. With the aim to confirm encapsulation of drug and magnetite particles in PLGA matrix, FTIR spectroscopy was used. The IR spectra of samples were measured using AVATAR 330 FT-IR Nicolet spectrometer by means of the KBr pellet method. In this method, the solid sample was finely pulverized with pure, dry KBr, the mixture was pressed in a hydraulic press to obtain a transparent pellet, and the spectrum of the pellet was measured. Finally, colloidal stability of PLGA composite nanosphere dispersions in the presence of added electrolyte was investigated via turbidimetry. Typically, 100 μl of dispersion was added to 3 ml of NaCl solution (from $1x10^{-4}$ to 4 $mol.dm^{-3}$). The samples were allowed to stand for 40 min at ambient temperature and their absorbance was measured over the range 400 to 700 nm with step 10 nm [5]. The gradient, *n*, of the straight line plot of log absorbance against log wavelength was calculated. The critical aggregation concentration (CAC) of added electrolyte was determined for each dispersion from the sharp transition point in plots of *n* against added electrolyte concentration.

3. Results and discussion

A magnetic PLGA nanospheres with encapsulated Taxol (PLGA/TAX/MFPEG) were prepared by the modified nanoprecipitation method as described in Materials and methods. After the sphere formulation, the samples were characterised by SQUID magnetometer from 4.2 K to 300 K, SEM, PCCS technique, FTIR and DSC measurements. SQUID measurements (data not shown) showed superparamagnetism of prepared PLGA

composite NPs with a blocking temperature of 160 K and saturation magnetization 1.4 mT. SEM (Fig. 1) was used to determine the morphology and particle size of the magnetite and drug loaded polymeric nanospheres.

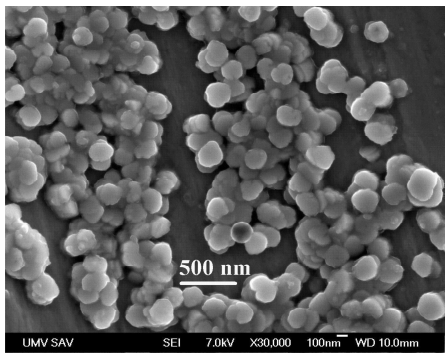

Fig. 1 SEM image of Taxol loaded magnetic PLGA nanospheres.

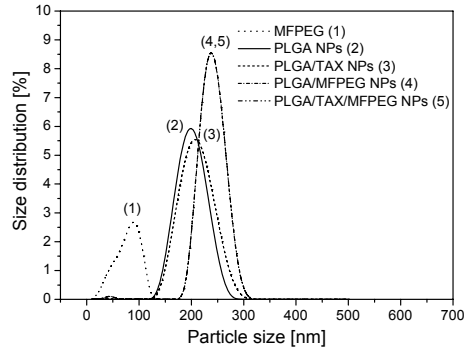

Fig. 2 The particle size distribution of PLGA composite nanospheres and pure MFPEG.

The surface was primarily smooth, although some roughness could be identified in certain areas of some spheres. The mean diameter of all PLGA samples was 200–250 nm and the results were in good agreement with the results obtained from the PCCS technique (Fig. 2). The values of the mean nanosphere diameter are summarized in Table 1.

DSC was used to determine the solid-state solubility of the drug in the polymer at the drug melting point and the thermodynamic properties of the drug-polymer system. Values of melting enthalpy ΔH_m (J/g) were plotted as a function of Taxol loading percentage (Fig. 3). The inset shows the DSC traces of the PLGA NPs with variable amounts of the added drug up to 37.5 % wt (m_{TAX} /(m_{TAX} + m_{PLGA})). In these thermograms, endothermic peak for polymer (I) was appeared around 58°C and for the drug (II) around 225°C. At the melting temperature of Taxol, the observed endothermic process corresponds to the melting of the residual undissolved drug. The X-intercept, provided by linear regression of the data, yields the solid-state solubility of Taxol in PLGA at 15 %. The solid state solubility of the drug in PLGA NPs is an important parameter which influences the drug encapsulation and release from NPs [6]. In Fig. 4 there are DSC curves of pure Taxol, physical mixture of Taxol + unloaded magnetic PLGA NPs, Taxol loaded magnetic PLGA NPs (PLGA/MFPEG/TAX NPs), blank PLGA NPs and magnetic particles loaded PLGA NPs (PLGA/MFPEG NPs). As seen in Fig. 4, we observed no peak in the temperature range of 70–250°C for blank PLGA NPS as well as for MFPEG/PLGA NPs and TAX/MFPEG/PLGA NPs. The endothermic melting peak of pure Taxol is only observed in the thermogram of the physical mixture and therefore, it could be concluded that Taxol in NPs was in an amorphous or disordered crystalline phase of molecular dispersion or a solid solution state in the polymer matrix after the production.

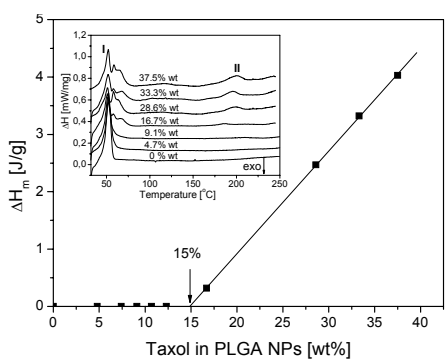

Fig. 3 The relationship between Taxol loading and the ΔH_m of the incorporated drug.

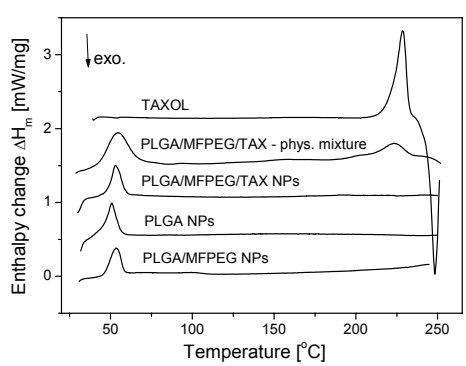

Fig. 4 DSC thermograms of prepared PLGA composite nanospheres and physical mixture.

Table 1: Summarized results of particle size distribution (PCCS analyse, SEM), colloidal stability (CAC) and toxicity (LD_{50})

Sample	*Particle size [nm]*		*CAC [mol/dm³ NaCl]*	*LD_{50}*
	PCCS	SEM		
MFPEG	65	60	0.095	400 mg Fe_3O_4/kg
PLGA NPs	195	198	1.6	221 mg PLGA/kg
PLGA/Taxol	204	208	1.2	226 mg PLGA/kg (at 11.3 mg TAX/kg)
PLGA/MFPEG	230	224	stable	174 - 198 mg PLGA/kg
PLGA/MFPEG/TAX	232	240	stable	154 mg PLGA/kg (at 7.7 mg TAX/kg)

With the aim to confirm the encapsulation of Taxol and MFPEG in PLGA matrix, FTIR spectroscopy was used. In Fig. 5, the infrared spectrum of PLGA/TAX/MFPEG NPs (d) shows a characteristic absorption band of PLGA spectrum (a) observed at 1759 cm^{-1} (C=O in carboxyl/ester group) with two shoulders corresponding to characteristic absorption bands of pure Taxol observed at 1736 cm^{-1} and 1713 cm^{-1} in the spectrum of pure Taxol (b). Comparing the spectra (c) and (d) an increased transmittance (reduced absorption band) can be seen at 1647 cm^{-1} when Taxol is encapsulated. This suggests that there is an interaction between the molecules of nanospheres and the molecules of Taxol involving the amide group. The band observed at 1244 cm^{-1} for pure Taxol (b) and physical mixture of Taxol and PLGA NPs (c) is also observed in the spectrum of PLGA/TAX/MFPEG NPs (d) at 1250 cm^{-1} but is weaker. These results confirm that Taxol was successfully encapsulated into PLGA NPs. The presence of absorption bands at 1560 cm^{-1} and around 590 cm^{-1} in spectrum (d), that correspond to oleate anions and magnetite, respectively, confirm the successful encapsulation of magnetite particles into PLGA NPs.

The stabilities of the various dispersions towards added electrolyte are indicated in the plots of *n* against sodium chloride concentration (Fig. 6). Apart from dispersion PLGA/MFPEG and Taxol encapsulated in magnetic PLGA Nps (PLGA/TAX/MFPEG), which remained stable at all added electrolyte concentrations studied, the other dispersions exhibited a clear critical aggregation concentration (CAC) of added sodium chloride. The values of CAC obtained for the dispersions are given in Table 1. The results measured with magnetic fluid MFPEG are also given for comparison.

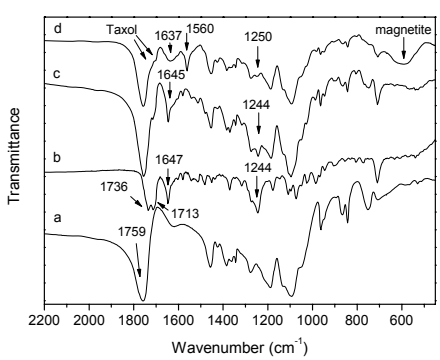

Fig. 5 FTIR spectra of a) pure PLGA NPs, b) pure Taxol, c) physical mixture of PLGA/TAX and d) Taxol loaded in magnetic PLGA NPs.

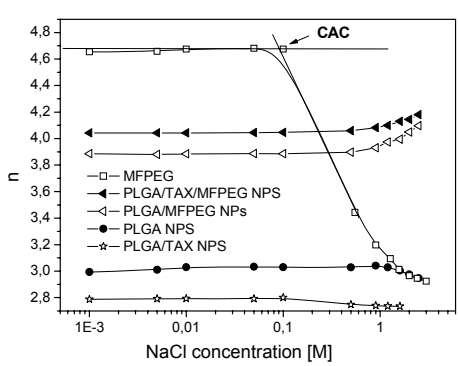

Fig. 6 Variation of *n* at 25°C as a function of NaCl concentration for pure MFPEG and PLGA composite nanospheres.

In vivo toxicity of PLGA composite NPs and magnetic fluid (as a blank) was evaluated in ICR mice after intravenous administration using Up and Down method (OECD 421). LD_{50} value of magnetic fluid MFPEG (conc. 100 mg Fe_3O_4/1 ml) determined in male mice was 400 mg Fe_3O_4/kg. LD_{50} value of PLGA nanoparticles (conc. 10 mg /1 ml) determined in male mice was 221 mg PLGA /kg. In the combination of NPs with magnetic fluid PLGA/MF PEG **(**100 mg PLGA/50 mg Fe_3O_4/10 ml) the PLGA was assumed as limit component and for this reason the LD_{50} is expressed in concentration of this component. LD_{50} was determined to be in the range of 174–198 mg/kg; doses of 174 and 198 were applied three times because the dose 174 mg/kg wasn't lethal for any of the animals, the dose of 198 mg/kg caused the death of all animals. The last experiment was done on the sample consisting of Taxol loaded magnetic PLGA NPs (100mg PLGA/50 mg Fe_3O_4/5mg Taxol). The dose of

154 mg/kg hasn´t been lethal. The results are also summarised in Table 1. The evaluation of cytotoxicity will be subject of the future experiments.

In conclusion we can say that we have prepared and characterized Taxol loaded magnetic PLGA NPs with spherical shape of mean diameter lower than 250 nm, which is a relevant size for intravenous administration. The prepared NPs have been found to have good stability in the presence of high NaCl concentration at 25°C, the toxicity of prepared samples declared 5 times higher value of lethal dose LD_{50} in comparison with pure Taxol (LD_{50} = 33 mg/kg) and showed significant response to external magnetic field which is useful for drug delivery systems for tumour treatment.

Acknowledgements This work was supported by the Slovak Academy of Sciences (grant No. 6166), Slovak Research and Development Agency under the contracts No. APVV- 26-026505 and APVV – 0347 - 06.

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Processing of stalled replication fork under thymine starvation and its relationship with thymineless death in *Escherichia coli*

I. Salguero Corbacho, E. Guarino Almeida, I. Soriano Moruno, A. Jiménez-Sánchez, and **E. C. Guzmán***

Departamento de Bioquímica, Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, 06080-Badajoz, Spain.

*Corresponding author: e.mail eguzmac@gmail.com. Phone: +34 924289300 ext 6955

In this work we characterized the mechanism by which the stalled replication forks generated under depletion of deoxynucleotides (dNTP) are processed and the relationship of the fork processing with the viability of the cell. RFR occurred at the stalled forks generated by hydroxyurea treatment (chemical inactivation of NDP reductase), was impaired under incubation of *nrdA101* mutant at 42ºC (structural inactivation of NDP reductase), and did not take place under thymine starvation. Viability experiments confirmed RFR model predictions and supported that thymineless death (TLD) is related with the processing of the stalled forks. Furthermore we show that TLD conditions suppressor generated stalled forks processed by RFR. We suggest is that the connection between DNA replication and TLD is through the fate of the stalled replication forks when thymine is removed.

Keywords replication, TLD, DSB, RFR, NDP reductase

1. Introduction

Impairment of replication fork progression is a serious threat to living organisms and a potential source of genome instability [1, 2]. Several strategies have been proposed for the processing of inactivated replication forks. Most of these require the action of recombination proteins, with different proteins being implicated, depending on the cause of the fork arrest [3]. In several replication mutants, the stalled forks generated upon inactivation of the mutant enzyme are reversed and result in the formation of a Holliday junction (HJ) adjacent to a DNA double strand end, a reaction called 'replication fork reversal' (RFR) (Fig.1A) [4]. In a *rec* proficient background this intermediary could be processed without generating DNA double-strand breaks (DSBs) by using the recombination proteins RecBCD, RecA, and by the HJ-specific resolvase RuvABC (Fig.1 B) [5]. In contrast, in the absence of RecBCD activity (Fig. 1C), resolution of the RFR-produced HJ is done by RuvABC resolvase and leads to fork breakage [6]. These particular DSBs are dependent on RuvABC activity in a *recB* deficient background. In this work we did a comparative study of the ability of the replication forks to be reversed after they have been stalled by depletion of DNA precursosors, deoxyribonucleotides (dNTP). The depletion of dNTP was achieved by inactivation of NDP reductase and by thymine starvation. Inactivation of NDP reductase was attained in two ways: by addition of hydroxyurea (Hu), which is a specific inhibitor of NDP reductase activity [7], or by the incubation of an *nrdA101* thermosensitive mutant strain at 42ºC.

NDP reductase is the only specific enzyme required for the enzymatic formation of dNTP, and has been proposed to be a structural component of the replication hyperstructure in *E. coli* [8,9]. It is a 1:1 complex of two subunits called proteins R1 and R2, coded by genes *nrdA* and *nrdB* respectively [10]. For many years it has been known that thymine auxotrophic microorganisms undergo cell death in response to thymine starvation, the so-called thymineless death (TLD) [11]. This phenomenon has been researched for over five decades, but the molecular mechanism remains an enigma. Our proposal is that TLD is related with the fate of the stalled replication forks generated when thymine is removed.

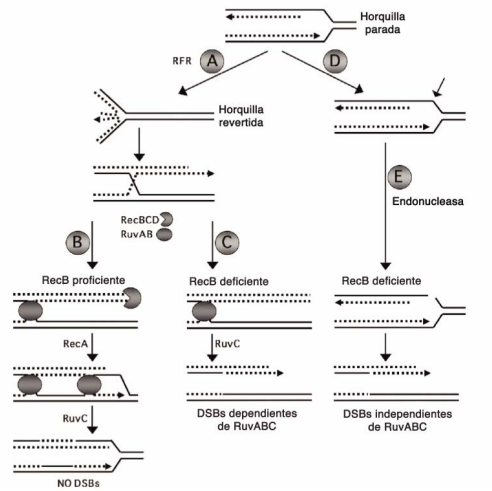

Figure 1. The fate of the stalled forks. In the first step (A), the replication fork is arrested, causing fork reversal. The reversed fork forms a HJ (two alternative representations of this structure are shown – open X and parallel stacked X). In Rec^+ cells (B), RecBCD initiates RecA-dependent homologous recombination, and the resulting double HJ is resolved by RuvABC. In the absence of RecBCD (C), resolution of the HJ by RuvABC leads to DSBs at the stalled replication fork. Alternatively, the replication fork is arrested without being regressed (D) and it is susceptible to be cut by an endonuclease, generating DSBs at the stalled replication fork (E). Continuous line (parental chromosome); dashed lines (newly synthesized strands); disk (RuvAB); incised disk (RecBCD).

2. Results and Discussion

2.1. Experimental approach

In this work we have studied the fate of the replication forks stalled using different ways of depleting DNA precursors. To verify whether the RFR process is occuring after a treatment, a *recB* deficient background should be used. According to the RFR model, the occurrence of this process at the stalled forks can be verified by testing whether there is an increase of DSBs in a *recB* deficient background, and determining whether these DSBs are dependent on RuvABC resolvase activity by measuring the amount of DSBs in a *recB* and *recB ruvABC* deficient background (Fig. 1 C) [6]. The occurrence of RFR at the stalled forks has been verified by this system in several replication mutants [4]. If RFR does not take place at the stalled fork, at least two situations may arise. In one hand, there would be an increase of DSBs independent of RuvABC activity and generated by another unknown endonuclease (Fig.1E) [1]. On the other hand, there would be no increase in the amount of DSBs probably because the stalled forks are not susceptible to the endonuclease activity, and the restarting of the forks would take place without the generation of fork breakage [4].

2.2. Starvation of DNA precursors induces DSBs

Cultures of strains JK626 (*thyA arg his recB258::Tn10*) and the isogenic *nrdA101* counterpart JS628, were grown at 30ºC in M9 minimal medium containing 5 µg/ml thymidine, 5 µCi/ml [*methyl*-^{3}H] thymidine (100 Ci/mmol), 20 µg/ml of required aminoacids and 0.2% of casaminoacids. By the time the cultures reached 0.2 OD_{450nm} one portion of each culture was treated with Hu 50 mM (freshly prepared at 1M in MM9), and a second portion in the case of the strain JK626 (*recB*) was thymidine starved at 30ºC by collecting the cells on a Millipore filter, and washing and resuspending them in M9 medium free of thymidine. To achieve the thermal inactivation of NDP reductase, one portion of the culture of JS628 (*nrdA101 recB*) strain growing at 30ºC was transfered to 42ºC. To determine the extent of DSBs, the amount of linear DNA was quantified before beginning the treatment, and after two hours of treatment for Hu addition and for 42ºC incubation, and after 30 min for the thymidine starvation treatment.

Cells labeled with [*methyl* ^{3}H]-thymidine were gently lysed in agarose plugs and their DNA was analyzed by pulse field gel electrophoresis (PFGE), in which only linear chromosomes enter the gels while circular molecules remain in the wells [2, 6].

The results show that inactivation of NDP reductase by Hu addition increases by up to twofold the amount of linear DNA in the strain JK626 (*recB)* and JS628 (*nrdA101 recB*), indicating the increase of DSBs (Table 1). However, when we tested whether incubation of the strain JS628 (*nrdA101 recB*) at 42ºC led to DSBs formation, we only found a slight increase of DSBs relative to 30ºC (Table 1, compare JS628 at 30ºC and at 42ºC). To investigate the occurrence of DSBs at stalled forks generated by TTP depletion without altering the NDP reductase, we measured the amount of linear DNA in JK626 (*recB)* under thymine starvation using a similar approach to that performed with the other conditions. DNA breakage has been observed under thymine starvation, but whether DSBs occur has been controversial. We found induction of DSBs in the strain JK626 (*recB*) after 30 min of thymidine starvation (Table 1).

Table 1. Amount of linear DNA in nrdA+ and nrdA101 strains under dNTP depletion conditions. Data are expresed in % linear DNA (mean±SD)

Strain	*Relevant genotype*	*30ºC*	*42ºC*	*+Hu*	*-TdR*
JK626	*nrdA+recB*	15.18±2.83	13.97±7.10	30.33±3.20	24.14±7.12
JK707	*nrdA+ ruv recB*	6.74±2.60	2.63±0.70	3.68±2.10	26.26±9.83
JS628	*nrdA101 recB*	24.79±7.05	32.09±6.02	40.88±2.81	-
JS705	*nrdA101 ruv recB*	5.94±2.19	3.34±2.28	5.55±0.93	-

2.3. Stalled forks created by thymidine starvation are not reversed

In order to test whether these DSBs resulted from the action of RuvABC, PFGE was performed with the strain JK707 (*thyA arg his recB258::Tn10 ΔruvABC::Cm*) in the presence of Hu or thymidine starvation, and with the isogenic strain JS705 *nrdA101* in the presence of Hu or after incubation at 42ºC. The level of linear DNA dramatically decreased in the presence of Hu or the incubation at 42ºC (Table 1), indicating that the DSBs induced by dNTP depletion resulted from the RuvABC resolvase activity in a *recB* deficient background. Therefore we conclude that RFR is induced at the stalled forks generated by depletion of dNTP. Nevertheless, the incubation of *nrdA101* strain at the restrictive temperature induced only a small increase of RuvABC-dependent DSBs. This would indicate that stalled forks generated under thermal inactivation of the NDP reductase have much lower propensity to be regressed and cut by RuvABC resolvase than the stalled forks generated under chemical inactivation of the enzyme. Surprisingly, we found that the DSBs level was not reduced under thymidine starvation in *recB ruvABC* strains (Table 1). Consequently these DSBs were not dependent on RuvABC resolvase activity as they occurred under RuvABC inactivation, indicating that replication forks stalled by thymine starvation do not undergo RFR process. The DSBs found under this treatment could have been generated either (i) by direct endonucleolytic cleavage of single strand breaks at or near stalled forks (Fig. 1D), or (ii) by cleavage of single strand breaks at places not related with replication fork, or (iii) by both.

2.4. Reversed replication forks prevent lethality

In addition to the formation of RuvABC dependent DSBs in a *recB* deficient context, RFR model would imply differences on the viability of *rec*-deficient strains under Hu treatment and thymidine starvation. In one hand, if RFR occurred after Hu addition it would be expected: 1) a lethality in the *recB* mutant strain due to the unrepaired DSBs, and 2) a suppression of this lethality by the inactivation of RuvABC resolvase in a *recB ruvABC* deficient strain. On the other hand, if RFR was not taking place under thymidine starvation, TLD should not be alleviated in the *recB* mutant strain by the deficiency of the RuvABC resolvase; as DSBs induced by thymidine starvation are not avoided in a *recB ruvABC* deficient strain.

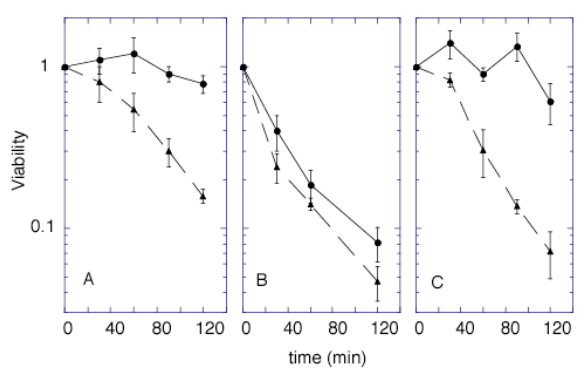

Figure 2. Effect of Hu addition (●) and thymidine starvation (▲) on the viability of (A) the *rec*+ strain, JK607; (B) the *recB* deficient strain, JK626, and (C) *recB ruvABC* deficient strain, JK707. Bold lines indicate the standard deviation from at least four independent experiments.

Viability experiments were performed by growing the strains JK607 (*rec*+), JK626 (*recB*) and JK707 (*recB ruvABC*), up to 0.2 OD and treated by Hu addition or by thymidine starvation in the same experimental conditions as PFGE experiments. At indicated time intervals 0.05-ml aliquots were removed, appropriately diluted, and plated on rich medium plates for determination of viability. We found that the viability of the *recB* deficient strain to be highly sensitive to Hu addition and thymidine starvation compared with wild type (Fig. 2A, B), as expected by the increase of un-repaired DSBs in the absence of RecBC activity. This detrimental effect was recovered by the inactivation of RuvABC resolvase only in the case of Hu addition but not under thymidine

starvation (Fig. 2C). These results support that RFR occurs when DNA replication was inhibited by Hu treatment, but not when it was performed by thymidine starvation.

2.5. Reversed replication forks prevent TLD

Recently, Morganroth and Hanawalt have observed that TLD occurs under Hu addition [12]. According to our proposal it could be expected a supression of TLD under Hu addition, but it has to be pointed out that our prediction would be only in the case the stalled fork underwent RFR under these conditions. In the mentioned work Hu addition was performed at the same time as the culture was thymine starved, and under these conditions the fate of the stalled forks is, at this moment, unknown. In this work we performed the experiments separating in time both treatments. In one hand, Hu was added first, in order to generate reversed stalled replication forks, and five minutes later the culture was starved for thymidine in the presence or absence of Hu (Fig. 3A). In the other hand, the culture was starved for thymidine for 30 minutes first, in order to generate stalled replication forks no-reversed and then the culture was treated with Hu in the presence or absence of thymidine (Fig. 3B). These results show that TLD was supressed only by the addition of Hu to the culture prior to the starvation of thymidine, supporting that the proccessing of the stalled forks would have an important role in TLD.

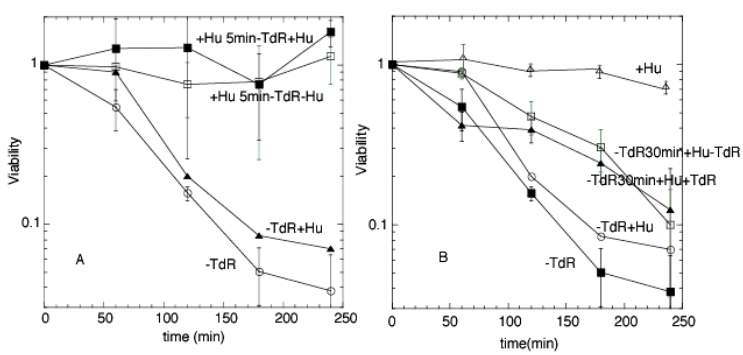

Figure 3. Viavility of rec+ strain JK607 under thymidine starvation and Hu addition. (A) Effect of 5 min of Hu addition prior to thymidine starvation in the presence or absence of Hu. (B) Effect of 30 min of thymidine starvation prior to Hu addition in the presence or absence of TdR. Bold lines indicate the standard deviation from at least four independent experiments.

Knowing that addition of rifampicin prevents TLD [11], we determined the origin of the DSBs generated under thymidine starvation in the presence of rifampincin. The results (Table 2) showed that the mayority of the DSBs under this condition were originated by RuvABC endonuclease, indicating that the inhibition of transcription permited the reversion of the replication forks stalled by thymidine starvation, and furthermore that there is a relationship between the fate of the stalled replication forks and the effect of the treatment on the cell viability.

Table 2. Amount of linear DNA in nrdA strains under thymidine starvation in the presence of rifampicin. Data are expresed in % linear DNA (mean±SD)

Strain	*Relevant genotype*	*30ºC*	*-TdR*	*-TdR +Rif*	*+Rif*
JK626	*nrdA+recB*	15.18±2.83	24.14±7.12	24.50±2.05	13.97±1.31
JK707	*nrdA+ ruv recB*	6.74±2.60	26.26±9.83	10.46±3.11	3.82±0.98

3. Concluding remarks

The present data show that stalled replication forks display a differential capacity to undergo RFR after depletion of deoxynucleotides brought about in three different ways. Replication fork reversal occurred at the stalled forks generated by Hu addition, was impaired with thermally inactivated NDP reductase, and it did not take place with thymine starvation. Besides the absence of RFR at the replication forks under thymine starvation, the occurrence of DSBs independent of RuvABC endonuclease needs to be explained. We suggest that these DSBs (or at least a number of them) were originated by endonucleolytic cleavage at or near stalled replication forks (Fig.1 D, E), explaining the connection between replication forks stalled by thymine starvation and TLD proposed by Maaløe and Hanawalt in the 1960s. Our proposal is that this connection is not due to the replication process it self, as it has been shown not to be required [12], but through the fate of the stalled replication forks when thymine is removed.

Acknowledgements The support by grant BMC2002-00830 from the Ministerio de Educación y Ciencia and by grant 2PR04A036 from Junta de Extremadura are gratefully acknowledged.

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Targeting of exogenous β-carotene oxygenase into the chloroplast is essential for its efficient function in the microalga *Chlamydomonas reinhardtii*

I. Couso, M. Vila, J. Vigara and R. León*

Biochemistry Lab. Dpto. Química y Ciencia de Materiales. Universidad de Huelva. Avd. Fuerzas Armadas s/n. 21071Huelva. Spain.

*Corresponding author: e-mail: rleon@uhu.es . Phone: +34 959 219951

Carotenoids synthesis takes place in the chloroplast but is catalysed by enzymes encoded by nuclear genes. These enzymes are thus synthesised in the cytoplasm as precursor polypeptides with an amino-terminal extension, the transit peptide (tp), and targeted to their final location in the chloroplast. β-carotene oxygenase (BKT) mediates the addition of keto groups to the position C4 of β-ione-rings of carotenoids. We studied the effect of the transit peptide sequences of Rubisco small subunit (RbcS2), Ferredoxine (Fd) or Phytoene desaturase (Pds) from *Chlamydomonas* corresponding proteins and the absence of tp on the functionality of exogenous *bkt1* in transgenic *Chlamydomonas reinhardtii*. RbcS, Fd and Pds are targeted to the chloroplast stroma, intertylakoid lumen and tylakoid membrane respectively. Our results indicate that the presence of a chloroplastic transit peptide is essential for the efficient production of ketocarotenoids in transgenic strains of the microalga *Chlamydomonas reinhardtii.*

Keywords Bkt, carotenoids; microalgae, Chlamydomonas reinhardtii.

1. Introduction

Carotenoids are a group of over 600 isoprenoids which work as auxiliary pigments in photosynthetic organisms. They are also synthesised by some bacteria and fungi and can be found in chromoplasts of many flowers and plant fruits. Ketocarotenoids, differently from other carotenoids which are found in most green tissues, are limited to certain microorganisms, such as the chlorophyte *Haematococcus pluvialis*, which is the main natural source of the ketocarotenoid astaxanthin. Ketocarotenoids exhibit superior antioxidant properties than β-carotene and have important commercial applications as feed supplements for fish aquaculture and as nutraceutical for human nutrition. The conversion of β-carotene into astaxanthin in the green alga *H. pluvialis* is catalysed by the enzymes β-carotene oxygenase (BKT) and carotene-β-hydroxylase (CHYB) (Fig.1) BKT enzyme mediates the addition of keto groups to the position C4 of β-ione-rings of carotenoids. While CHYB is present in all carotenogenic tissues and is responsible for the conversion of β-carotene into zeaxanthin, most higher plants and microalgae do not posses the β-carotene ketolase (BKT) activity and consequently lack astaxanthin or other ketocarotenoids. Expressing *H. pluvialis bkt* gene in *E. coli* [1; 2], cyanobacteria [3], higher plants [4-7] and in the unicellular microalga *Chlamydomonas reinhardtii* [8] has allowed the production of ketocarotenoids that were not found in the corresponding wild untransformed strains. Carotenoids synthesis takes place in the chloroplast but is catalysed by enzymes encoded by nuclear genes. These gene products are synthesized on cytosolic ribosomes as precursor polypeptides with an amino-terminal extension and post-transcriptionally imported into the chloroplasts by a transit peptide (tp) which interacts with the chloroplast transport apparatus and leads to translocation of the protein across the chloroplast envelope [9].

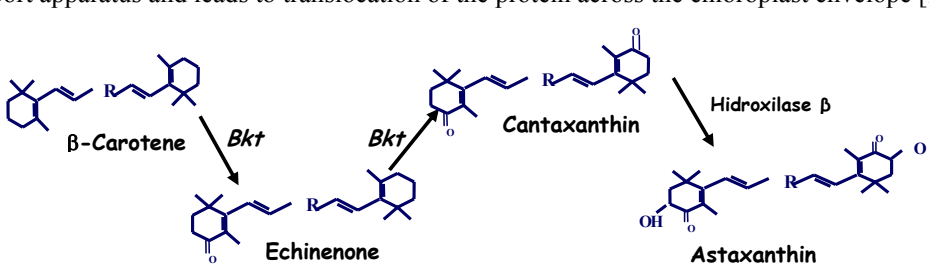

Fig. 1: Carotenoids pathway from β-carotene to Astaxanthin

To check the importance of the presence of a transit peptide on the expression of *bkt1* gene in transgenic *Chlamydomonas reinhardtii* we studied the effect of the transit peptide sequences of Rubisco small subunit (RbcS2), Ferredoxine (Fd) or Phytoene desaturase (Pds) from *Chlamydomonas* corresponding proteins on the functionality of exogenous *bkt1*. RbcS2, Fd and Pds are targeted to the chloroplast stroma, interthylakoid lumen and thylakoid membrane respectively. We analysed the phenotype of the transformant strains obtained by expressing the different types of transit peptide and the *bkt1* gene and compared it with Chlamydomonas transformed with BKT not preceded by any transit peptide.

2. Materials and Methods

2.1. Microorganisms and standard culture conditions

H. pluvialis (SAG192-80) which was kindly provided by the Plant Biochemistry and Photosynthesis Institute (CSIC, Seville) and photoautotrophically cultured in the medium was the described by Seuoka et al. in 1967 [10]. *Chlamydomonas reinhardtii* cell-wall deficient strain 704 (Cw15, Arg7, mt+) was kindly provided by Dr Roland Loppes [11] and cultured photomixotrophically in TAP medium at 25ºC under continuous white light irradiation. *Escherichia coli* (DH5α) was cultured in LB medium.

2.2. RNA extraction and reverse transcription-PCR

Total RNA was isolated from *H. pluvialis* cultures subjected to nitrogen starvation and high light intensity (400 $\mu Em^{-2}\ s^{-1}$) as previously described [8]. cDNA was synthesized from total RNA with SuperScript II RNaseH-reverse transcriptase (Invitrogen) and used as substrate for PCR reactions. The PCR amplification was performed from 2µl of the RT reaction mixture in a total volume of 50 µl containing 20 pmol of each primer, 0.2mM dNTPs, 1Upfu *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 5µl of specific 10× buffer (containing 2.5mM $MgCl_2$), and 1% dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96 ◦C, 0.5 min at 42 ◦C, and 1.5 min at 72 ◦C for 30 cycles.

2.3. Construction of expression vectors for Chlamydomonas

Plasmid pSI104PLK was obtained from plasmid pSI103 [12] as previously described [8]. The β-carotene oxygenase cDNA (*bkt1*) was amplified by RT-PCR based on the sequence (GeneBank accession no. X86782) published by Harker and Hirschberg in 1997. The obtained PCR product (1.4kb) was subcloned between the *Xho*I and *Pst*I restriction sites of the *Chlamydomonas* expression vector pSI104PLK. DNA fragments encoding the desired transit peptides were synthesized by Genescript Co. (NJ, USA) and fused in the same reading frame to the *bkt1* gene to drive the encoded protein into the chloroplast.

2.4 Nuclear transformation of *Chlamydomonas reinhardtii*

Transformation was carried out using the glass-bead method of Kindle [16]. The transformed cells were pelleted and spread onto TAP medium with paramomycin (30 $\mu g\ ml^{-1}$). Colonies were visible after 4 or 5 days.

2.5. Analytical determinations

Separation and chromatographic analysis of pigments was performed in a Merck Hitachi HPLC equipped with a UV-Vis detector as described by Young et al. [17]. Pigments detection was carried out at 450 nm, Pigments standards were supplied by SIGMA or DHI (Hoersholm, Denmark). The Ketocarotenoid was identified using an Agilent 1100 series chromatograph equipped with a diode-array and a mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA). Dry weight was determined by filtering an exact volume of microalgae culture (30 ml) on pre-tared glass-fiber filters (1µmpore size). The filter was washed with a solution of ammonium formate (0.5 M) and dried at 100º C for 24 h. The dried filters were weighed in an analytical balance and the dry weight calculated by difference.

3. Results and Discussion

3.1 Choice of the different transit peptides

We chose transit peptides from Rubisco small subunit (RbcS2), Ferredoxin (Fd) and Phytoene desaturase (Pds), which are targeted to the chloroplast stroma, interthylakoid lumen and thylakoid membrane respectively, to direct the exogenous carotenogenic enzyme β-carotene oxygenase into its final location in the chloroplast. The transit sequences of the *Chlamydomonas* RbcS2 and ferredoxin are small peptides with 32 amino acids, well known at both primary sequence and structural levels [13; 14]. The transit peptide corresponding to the phytoene desaturase preprotein of the same microalga is not so well defined. We have estimated the probable transit peptide sequence from the predictions of the ChloroP programme (*www.cbs.dtu.dk/services/ChloroP*) and alignment among eukaryotic and procariotic PDS sequences (fig.2).

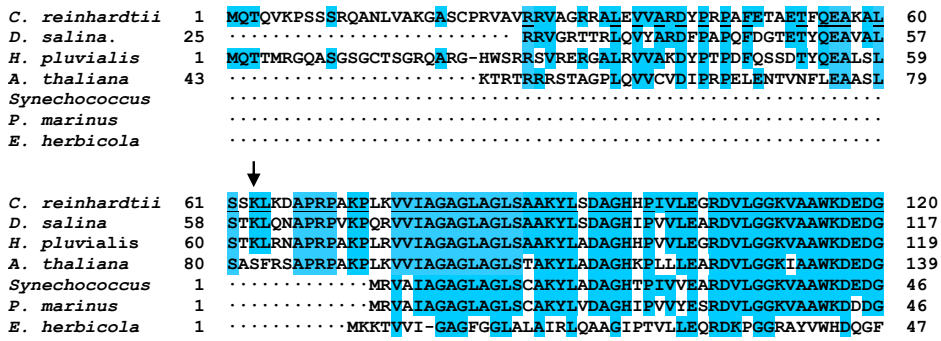

Fig. 2: Aligment of the first 120 aminoacids of Chlamydomonas Phytoene desaturase (PDS) with PDS of other organisms. Aminoacids identical to Chlamydomonas PDS sequence are shaded. Sequences conserved in the three microalgal strains studied are underlined. The arrow shows the probable chloroplast transit peptide cleavage site predicted by ChloroP program. Species and GenBank accession numbers are: *Chlamydomonas reinhardtii* PDS (X86782); *Dunaliella salina* PDS (ABB51091.1); *Haematococcus pluvialis* PDS (AAV37090.1); *Arabidopsis thaliana* (NP974545.1); *Synechococcus* PDS (ZP01122599.1); *Prochlorococcus marinus* PDS (NP895829.1); *Erwinia herbicola* CRTI (P22871).

The PDS precursor mRNA has been recently identified in *Chlamydomonas reinhardtii* [15] as a 564-aminoacids precursor polypeptide, of high similarity in sequence and length with the corresponding enzyme of other microalgae and higher plants, especially with PDSs of other microalgae such as Dunaliella and Haematococcus with 79% and 75% of identity. Homology of Chlamydomonas PDS is also very high with the crtP-encoded desaturases of cyanobacterium, such as Synechococcus and Prochlorococcus, with percentages of identical residues of 68%, but the length of these desaturases is significantly lower. The presence of a conserved region of about 73 aminoacids upstream the beginning of cyanobacterial PDS sequences suggests the possibility that this N-terminal region could correspond to the transit peptide. Aligment of the bacterial *Erwinia herbicola* PDS (CrtI) starts with Chlamydomonas PDS after the 76 residue also support this possibility, although homology is significantly lower in this case (36%). ChloroP(*www.cbs.dtu.dk/services/ChloroP*) predicts a cleavage site at amino acid 62 of Chlamydomonas PDS. With this data and the conclusions from the alignment of PDS sequences we have chosen the first 62aa of the PDS preprotein as transit peptide.

3.2. Cotransformation of Chlamydomonas with pSI103 and the constructed vectors pSI104PLK- *bkt1* and pSI104-tp-*bkt1*

The *bkt1* gene was isolated from *H. pluvialis* and inserted in the Chlamydomonas expression vector pSI104LK, as previously described [8]. This vector has the constitutive promoters of Heat shock protein 70A (HSP70A) and rubisco small subunit (RbcS2) and the rubisco small subunit terminator region (Fig.3a).

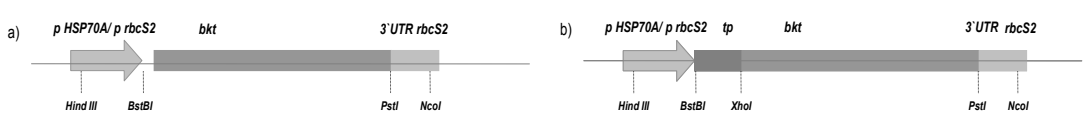

Fig. 3: Representation of the constructions used to express the *bkt1* gene in *Chlamydomonas: pHSP70A,* Heat shock protein 70A promoter*; prbcS2*, ribulose 1, 5 biphosphate carboxylase small subunit promoter; *ter rbcS2*, ribulose 1,5 biphosphate carboxylase small subunit terminator region; *tp*, transit peptide; *bkt*, β-carotene ketolase.

Beside this, three other constructions were designed using the DNA sequences encoding the three transit peptides chosen (See section 3.1) flanked by the *BstBI* and *Xho*I restriction sites (Fig. 3b). They were synthesized and fused in the same reading frame to *bkt1*, previously inserted in the polylinker region of the plasmid pSI104PLK. *C. reinhardtii* was co-transformed by these new constructions, and the plasmid pSI103 that carries the *AphVIII* gene that conferees resistance to paramomycine [12]. Paramomycine-resistant transformants were screened by PCR to confirm *bkt1* integration in their genome. About 40% of the paramomycine-resistant microalgae transformed with any of the four constructions had integrated the *bkt1* gene in their genome.

3.3 Carotenoids analysis of the four different Chlamydomonas transformants strains

The positive co-transformants selected by PCR were grown in TAP liquid medium and transferred to nitrogen-free medium where they were incubated during 48 h and analysed for their pigments content by HPLC. In those transformants in which the *bkt1* gene was preceded by the sequences corresponding to Fd or the RbcS2 the presence of a new carotenoid, identified as 4-ketolutein or its isomer 4-ketozexanthin in base to its retention time (8.4 min), its typical ketocarotenoid absorption spectra (Fig 4) and its mass spectra (*m*/*z* 583.4 (*M*+H)), was detected.

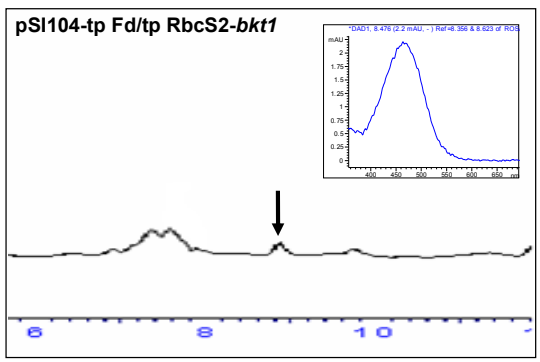

Fig. 4: Detail of the typical HPLC chromatogram of a pSI104PLK-tp Fd/tp RbcS2-*bkt1* transformant and the corresponding UV/Vis spectrum with a 470nm maximum.

This new pigment was not found in the strains transformed with the Pds transit peptide sequence fused to the *bkt1* gene or with the *bkt1* gene without any transit peptide. In these cases the carotenoid profile was very similar to that of the control untransformed cells. These results indicate that the presence of a chloroplastic transit peptide is essential for the efficient production of ketocarotenoids in transgenic strains of the microalga *Chlamydomonas reinhardtii.* The fact that the first 62 aminoacids of the PDS preprotein did not allow adequate expression of the *bkt1* gene makes us to think that the real transit peptide is longer, as suggests the comparison between prokaryotes and eukaryotes PDS sequences (Fig 2).

Acknowledgements: We want to thank the Spanish Ministry of Education and the Junta de Andalucía by financial support (AGL2004-08215-C03-01; PAI-CVI214) and Dr. E. Fernandez who kindly provided us the plasmid pSI103.

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The analysis on different SigB concentration in *Staphylococcus aureus* ~High SigB accumulation enhances biofilm formation~

Sayaka L. Takeshita*, Toshie Hidaka, Toshiko Ohta, and Kazuya Morikawa

Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, 305-8575 Japan

*Corresponding author: e-mail: morikawa@sakura.cc.tsukuba.ac.jp, Phone & Fax: +81-29-853-3928

Alternative sigma factor, SigB, plays an important role in the survival under stressed condition. In previous study, we found that SigB concentration is varied among strains, and methicillin resistant strains tend to have higher SigB concentration and the same amino acid substitutions. In addition, difference of SigB concentration correlated with the tolerance for ultraviolet irradiation. Here, we further examined the significance of the high SigB concentration. Interestingly, it affected the initial attachment to the surface pretreated with human plasma and also the biofilm formation. In addition, we found that the high SigB protein level is not attributed to PBP2'.

Keywords sigma factor; SigB; *Staphylococcus aureus*; biofilm formation

1. Introduction

Staphylococcus aureus, a natural inhabitant of skin and mucous membrane, is an important human pathogen causing hospital infection. The methicillin-resistant *S. aureus* (MRSA) can be defined as the strain carrying the *mecA* gene encoding penicillin-binding protein 2' (PBP2'), which has the reduced affinity to β-lactams [1].

Sigma factor is the promoter recognition subunit of the RNA polymerase, and is required in the site-specific transcription initiation [2]. SigB controls the expression of a variety of genes responding to environmental stresses and is requires for resistance to stresses including antibiotics [3-7]. Normally, the anti-sigma factor, RsbW, binds SigB to inhibit the SigB activity [8].

Previously we showed that SigB concentration is different among strains. In spite of a variety of roles of SigB in the general stress response, the phenotypic difference due to the different SigB concentration was not detected except for the UV tolerance [9]. In this study, we found additional phenotypes affected by the SigB concentration.

Concerning with the reason for the high SigB concentration, we previously found that there are mutations generating three amino acid substitutions (I/V, D/N, Q/K) in SigB, and demonstrated that D141N or both of I11V and D141N is essential. However, this is not sufficient, and the additional factor(s) affecting the SigB protein level has been postulated [9]. Based on the fact that SigB concentration of MRSA is higher than that of MSSA [9], we examined the correlation between the SigB concentration and the expression of PBP2'. Our results clearly showed that there is no relationship between SigB concentration and PBP2', suggesting that SigB high accumulation is attributed to the mechanism other than the expression of PBP2'.

2. Results

2.1 Difference of SigB concentration affects biofilm formation

In order to clarify the significance of the high SigB concentration, we examined some phenotypes that is controlled by SigB. The SigB concentrations of mutant strains used in this study are shown in Fig. 1 [9]. r408 is the parental strain having VNK-type SigB. Mutant strains carrying VNK-type SigB (N) and VNQ-type SigB (C3) have similar SigB concentrations to r408. C1 having IDQ-type SigB and C2 having VDQ type SigB have lower SigB concentrations.

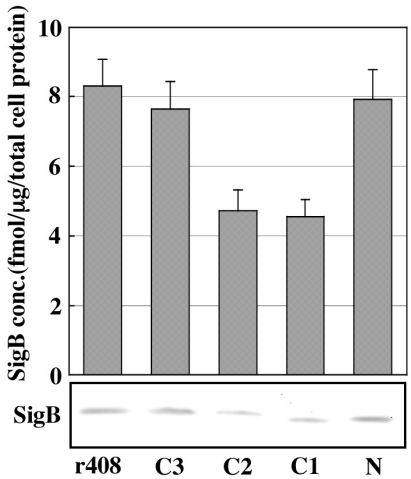

Fig. 1 SigB concentration in the mutants carrying different SigB amino acid substitutions [9]. SigB concentration was detected by quantitative Western-blot analysis (lower panel). The calculated SigB concentrations are indicated as fmol/μg protein with standard deviation (upper panel).

The biofilm forming ability of amino acid substitution mutants were compared by the method described previously [10]. The growth of cells was not significantly different among mutant strains: OD_{600} of r408 =1.059, N =1.030, C1 =1.070, C2 =1.074 and C3 =1.103. In contrast, the biofilm formed by C1 and C2 was less abundant than control strains (Fig. 2a).

We further examined the initial attachment by the method previously described [11] (Fig. 2b). The attachment of C1 was 41.2 +/- 21.3 % compared with r408, while that of the control strain, N1, was 93.8 +/- 22.3 % (Fig. 2b). These results suggest that high SigB accumulation can stimulate the biofilm formation possibly via the increase of the initial attachment.

Both DNase and autolytic activities, which might affect the biofilm state, were not different among these strains (data not shown).

2.2 The expression of PBP2' is not related to the high SigB protein level

Though N315 (Pre-MRSA) has a VNK-type SigB, its protein level is lower than other MRSA isolates, suggesting that additional factors also affect the SigB protein level [9]. In N315, the expression of PBP2' is normally repressed and is induced by antibiotic stress [12]. Therefore, we examined the possibility that SigB protein level may be elevated by the expression of PBP2'. The exposure of N315 cells to 1 μg/ml of oxacillin induced the expression of PBP2', but the increase of the SigB protein was not detected (Fig. 3a). Namely, PBP2' cannot elevate the SigB protein level.

Figure 3b substantiate this finding. The *mecA* expressing plasmid (pKIL-ts-mec) was introduced into N315ex (N315 strain that lost the *mecA* gene locus, SCCmec, [13]) and MSSA isolate s66 (carrying IDQ-type SigB, [9]). SigB protein level did not alter by the expression of the exogeneous *mecA* (Fig. 3b). Collectively, we concluded that high SigB protein level in MRSA isolates is not dependent on the expression of PBP2'.

3. Discussion

In this study, we showed that the difference of SigB concentration enhances the initial attachment (Fig. 2b) and the subsequent biofilm formation (Fig. 2a). The process of biofilm formation is divided into attachment phase, accumulation phase, maturation phase, and dispersal phase [14]. The high SigB concentration seems to enhance the earlier stages, because the amount of the mature biofilm was not significantly different among strains (Fig.2a 72h). MSCRMMs (microbial surface components recognizing adhesive matrix molecules) are important in the initial attachment stage [15], and SigB affects the expression of them such as Fibronectin-binding protein A [16]. In accumulation stage, polysaccharide adhesions (PIA) and DNA released by cell lysis effect biofilm development, and SigB is involved in the regulation of the PIA biosynthesis genes [17] and *cid* operon affecting cell lysis [18]. In addition, SarA that is regulated by SigB has been reported to contribute to biofilm-forming ability [19-21]. However, it is not known whether the quantitative difference of SigB has effects on the expression of these factors.

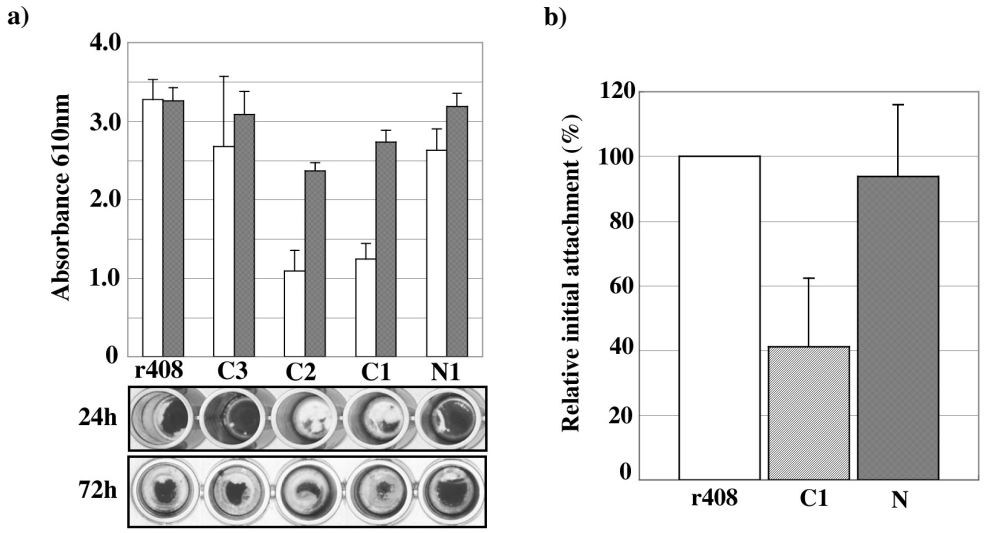

Fig. 2a) Biofilm formation by the SigB amino acid substitution mutants. Cells were grown for 24hour and 72hour in 96 well microtiter plate pretreated with human plasma. The crystal violet absorbed to the resultant biofilm was measured as described previously. Y-axis represents the averaged value of the absorbance at 610nm. 24hour:open bar. 72hour: gray bar
b) Quantification of initial attachment of r408, C1and N1 cells. After incubation at 37°C for 30 or 60 min, the wells were washed and the cfu/ml of the attached bacteria was measured as described [10].

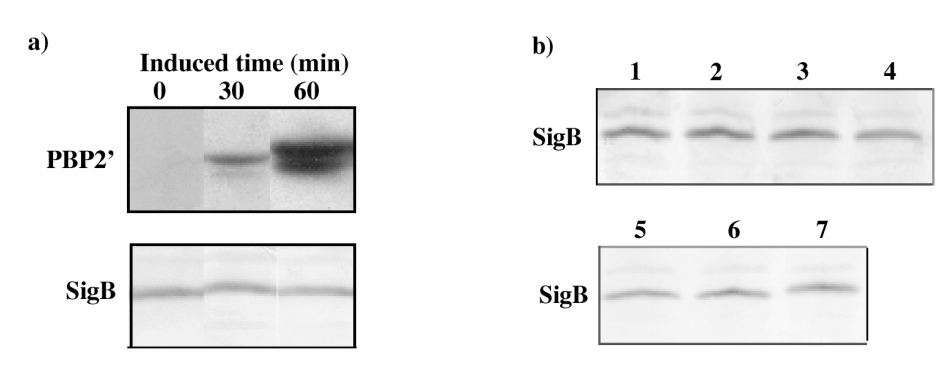

Fig. 3 Influence of PBP2' expression on the SigB protein level. **a)** The exposure to 1 μg/ml of oxacillin induced the expression of PBP2' (upper panel), but the SigB protein concentration was not altered (lower panel). **b)** The constitutive expression of *mecA* cannot elevate the SigB protein level. The *mecA* gene including the original promoter region (genome locus 44987-47132) of N315 was cloned into *Bam* HI-*Hin*d III site of the plasmid pKIL-ts [7], and checked by sequencing analysis. pKILts-mecA (lanes 3,6) or pKIL-ts-cat [22] (lanes 4,7) was introduced into *S. aureus* cells N315ex (lanes 2-4) and s66 (lanes 5-7) by electroporation. Introduction of pKIL-ts-mecA into N315 and s66 rendered cells resistant to oxacillin. Ten μg of total protein was submitted to the western blot analysis with anti-PBP2' monoclonal antibody and anti SigB-IgY [9]. Lane1: N315

The reason why all of MRSA isolates exhibit the high SigB level is still unknown. This study was carried out on the assumption that SigB concentration might be increased by the expression of PBP2' which was acquired through horizontal transfer. This expectation was excluded by two independent experiments: induction of PBP2' in pre-MRSA and introduction of *mecA* to MSSA. Considering that all of clinical MRSA isolates have VNK-type SigB [9], it might have played a role in the acquisition of the exogenous *mecA* gene.

Acknowledgements We are grateful to Dr. Teruyo Ito for providing PBP2' monoclonal antibody. We thank Gaichi Hasegawa and Yohei Kameda for technical support. This work was supported by Grant-in-Aid for Young Scientists B of the Japan Society for the Promotion of Science (to K.M.).

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Two new members of the *Tetrahymena* multi-stress-inducible metallothionein family: *T. rostrata* Cd/Cu metallothioneins

F. Amaro Torres, A. Martín-González and **Juan C. Gutiérrez***

Dpto. Microbiología III. Facultad de Biología. C/. José Antonio Novais, 2. Universidad Complutense de Madrid (UCM) 28040. Madrid. Spain.

*Corresponding author: e-mail: juancar@bio.ucm.es Phone: +34 913944968 Fax: +34 913944964

We report the cloning and characterization of two new metallothionein (MT) genes (cDNAs) isolated from the ciliated protozoa *Tetrahymena rostrata* (named as *TrosMTT1* and *TrosMTT2*). TrosMTT1 protein is included into the *Tetrahymena* CdMT subfamily, due to its similarity to *T. thermophila* MTT1 and its overexpression under Cd exposure, while TrosMTT2 is identified as a CuMT, due to its similarity to TpigMT-2 and its significant induction by copper. Both are also multi-stress-inducible genes because they are induced, at lower level, by other heavy metals. These two new *Tetrahymena* MTs complete the actual view of this protein superfamily, and corroborate the unique features of ciliate MTs.

Keywords metallothioneins; heavy metals; gene expression; *Tetrahymena rostrata*; ciliated protozoa.

1. Introduction

Metallothioneins (MTs) constitute a superfamily of ubiquitous, small (25-82 aa, <10 KDa), cysteine-rich proteins (7-21 conserved Cys residues) and lack aromatic amino acids and histidine. They are multifunctional proteins whose synthesis is enhanced by heavy metals and other environmental stressors. However, the most clear function of these proteins is the heavy metal detoxification [1,2]. MTs are found, not only throughout the animal kingdom, but also in other eukaryotes (protists, yeasts and higher plants) and in prokaryotic microorganisms (cyanobacteria) [3]. At present, in ciliated protozoa (exclusively in different *Tetrahymena* species), two MT subfamilies have been reported [2]; 7 CdMTs (subfamily 7a) and 4 CuMTs (subfamily 7b), which differ in the induction pattern by heavy metals (Cd or Cu) and the pattern of Cys residues clustering. Ciliate MTs present exclusive features [2, 4] with regard standard MTs, they are longer proteins (96-181 aa, 10-19 KDa), have a higher content in Cys residues (22-54) and aromatic amino acids are present in several of them [2]. We report here the cloning and characterization of two new MT genes (cDNAs) isolated from *T. rostrata* (Cd or Cu treated cell cultures). One of them has been identified as a CdMT due to both qRT-PCR results under different heavy metal treatments and Cys residues clustering or similarity to other *Tetrahymena* CdMTs, while the second one has been considered as a CuMT for similar reasons. A complete comparative analysis of all, at present, reported *Tetrahymena* MTs is showed and their potential biothecnological utilities are discussed.

2. Materials and Methods

Tetrahymena rostrata (ATCC-30770) was grown axenically in PP210 [2] medium at 28ºC. Prior to RNA isolation, ciliate cultures were exposed (24 hours) to different heavy metal stress conditions (concentrations used were approximately half the LC_{50} value); 27 μM Cd ($CdCl_2$), 80 μM Cu ($CuSO_4$. $5H_2O$), 870 μM Zn ($ZnSO_4$. $7H_2O$), 604 μM Pb ($Pb(NO_3)_2$), 421 μM Ni ($NiCL_2.6H_2O$) or 24 μM As ($NaHAsO_4$. $7H_2O$). Total DNA was isolated as described [5]. Total RNA was isolated using the RNAqueos™-4PCR kit (Ambion). All samples were treated with RNase-free DNase I (Ambion).

Convergent degenerate primers (MET1 and MET2) based on the sequence of the MT-1 gene of *T. pyriformis* [2] and designed primers (MTCu1 and MTCu3) from *TtherMTT2* gene were used to isolate *T. rostrata* MT genes by standard RT-PCR from Cd or Cu treated samples. The full-length cDNA sequence was obtained by RT-PCR coupled with RACE. Amplified RT-PCR products were cloned using the TOPO TA Cloning kit (Invitrogene). The cDNA synthesis, for quantitative real-time RT-PCR, was carried out using 5 μg RNA, oligo d(T) primer (5 μM). AMV reverse transcriptase (Roche) with RNase inhibitor (25 U) and 2.5 mM dNTPs in a total volume of 20 μl. cDNA samples were amplified in duplicate in 96 microtiter plates (Applied Biosystems). The α-tubulin gene was used as an endogenous control gene or constitutive cDNA. Real-time PCR reactions were carried out in an ABI PRISM 7700 real time PCR apparatus. All controls were negative (no template control and RT minus control). To calculate the relative change in expression we used the standard curve method. DNA sequences were determined using an ABI PRISM™ 377 DNA automatic sequencer (PE Applied

Biosystems), according to the dideoxy technique. Homology searches were performed using BLAST program at the NCBI website. Protein sequences were aligned using the T-coffee server, under default settings. Phylogenetic tree of the *Tetrahymena* MTs was constructed by using the UPGMA algorithm.

3. Results and Discussion

3.1 Structural characterization of two new MT genes in *Tetrahymena rostrata*

To isolate new metallothionein genes from *T. rostrata* we used standard RT-PCR of Cd or Cu treated samples. The obtained two full-length cDNAs were; *TrMTT2* of 398 bp in length, which included an open reading frame (ORF) of 237 bp and a 3´-UTR (including a polyA tail) of 161 bp, and *TrMTT1* of 522 bp, composed by an ORF of 342 bp, a 5´-UTR fragment of 64 bp and a 3´-UTR (including a polyA tail) of 116 bp. After alignment between cDNAs and genomic DNA coding sequences we conclude that both genes are intron-less, like all *Tetrahymena* MT genes at present report [2]. *TrMTT2* encodes a putative protein of 78 aa (8.2 KDa) which has 72.9% amino acid identity to TpigMT-2 (AF479586) and 58.3% identity to TtherMTT4 (AY660008), both are CuMTs. *TrMTT1* encodes a putative protein of 113 aa (11.5 KDa) which has 60.3% identity to TMCd1 *T. tropicalis* (ABM74559) (here named as TtropMTT1) and 59.2% identity to TtherMTT1 (AY061892), both CdMTs. Therefore, the inclusion of these new MTs as Cu- or CdMT is based on their highest identity percentages and their differential inductive responses to Cu and Cd (see 3.3 section). In Table 1, distribution of different types of Cys clusters among all, at present, reported *Tetrahymena* MTs is showed.

Table 1 Distribution of Cys clusters among *Tetrahymena* MTs

Cd-metallothioneins (subfamily 7a)

	TrosMTT1	TtropMTT1	TtherMTT1	TtherMTT3	TtherMTT5	TpyrMT-1	TpyrMT-2	All	All
Cluster type[1]								Total C	%C
CCC	4	6	6	2	1	4	6	87	31
CXCC	2	3	3	2	1	2	5	54	19
CXCXC	0	0	0	1	0	0	0	3	1
CCX	6	8	8	9	5	5	6	94	33
CXC	2	2	2	3	1	1	4	30	10
XXCXX	0	0	1	3	6	1	1	12	4
Total C	34	47	48	42	24	31	54	280	

Cu-metallothioneins (subfamily 7b)

Cluster type[1]	**TrosMTT2**	TtherMTT2	TtherMTT4	TpigMT-2	All (Total C)	All (%C)
CCC	0	0	0	0	0	0
CXCC	0	0	0	0	0	0
CXCXC	0	0	0	0	0	0
CCX	1	0	0	1	4	3.5
CXC	9	15	15	12	102	89.4
XXCXX	2	2	2	2	8	7
Total C	22	32	32	28	114	

[1]A cluster is defined here as any group of contiguous residues in which any two Cys are separated from one another by at most one non-Cys residue; every Cys belongs to a single cluster. XXCXX: unclustered Cys. Tros: *T.rostrata*, Tther: *T.thermophila*, Ttrop: *T.tropicalis*, Tpyr: *T.pyriformis*, Tpig: *T.pigmentosa*. Total C: total cysteine residues. Numbers in "total" and "%" rows or columns refer to Cys residues counts. The other numbers refer to Cys cluster counts.

The CCC cluster is almost exclusive of *Tetrahymena* CdMTs, does not exist in *Tetrahymena* CuMTs (Table 1), and are rare in standard MTs of other organisms. Likewise, CXCC and CXCXC clusters are not present in, at

present, reported *Tetrahymena* CuMTs (Table 1). The CCX, CXC clusters and unclustered Cys residues are the common Cys distribution in standard MTs, and, likewise, the only Cys organization in *Tetrahymena* CuMTs (Table 1). *Tetrahymena* MTs are considerably more rich Cys proteins (Table 1) than standard MTs (7-21 Cys) [1]. Considering that, in standard MTs, all Cys residues are involved in heavy metal binding and, therefore, the stoichiometry is $Cd_7(Cys)_{20}$ for CdMTs or $Cu_{12}(Cys)_{20}$ for CuMTs [6], we may conclude that TtherMTT1 might bind about 17 Cd atoms, TtherMTT3 (15 Cd), TherMTT5 (8 Cd), TpyrMT-2 (19 Cd), TrosMTT1 (12 Cd), TtropMTT1 (16 Cd) and TpyrMT-1 (11 Cd). This last theoretical data has been corroborated by experimental results [7], which showed that this CdMT may bind 12 Cd^{2+}/mol of protein.

Alignment of the TrosMTT1 protein sequence to subfamily 7a MTs of *Tetrahymena* reveals a remarkably regular and hierarchical modular organization, as it has been previously reported in *Tetrahymena* CdMTs [2]. TrosMTT1 presents a bi-modular structure (with a type 2 submodule of 19 aa) (Fig. 1). These modules appear to have evolved through the combinatorial accretion of repeat units of increasing length and complexity, built from two elementary types of motifs; C_2X_6 and CKCXXCKCCK (consensus). With only minor modifications, these motifs have been successively combined into submodules, modules and finally MTs [2].

Module (mod)		Linker	Type 1 submodule	Type 1 submodule	Type 2 submodule	Length
TpyrMT-1	mod 1	MD-KVNNN	CCGGENAKPCCTDPNSG	CCCVSETNNCCKSDKKE	CCTGTGEGCKCTGCKCCQ	52
TtherMTT1	mod 1	MD--KVNS	CCGGVNAKPCCTDPNSG	CCCVSKTDNCCKSDTKE	CCTGTGEGCKCVNCKCCK	52
TtherMTT1	mod 2	-----PQAN	CCGGVNAKPCCFDPNSG	CCCVSKTNNCCKSDTKE	CCTGTGEGCKCTSCQCCK	52
TrosMTT1	mod 1	MD---KNS	CCGGENAKPCCTDPNSG	CCCSSKTNNCCQSDTKE	CCTGTGPGCKCTSCKCCK	52
TrosMTT1	mod 2	-----PAT	CCGGDKAKPCCTDPNSG	CCCVSKTNNCCKPDTKQ	CCTGTGDACKCTGCQCCKQ	53
TtropMTT1	mod 1	MD---KVT	CCGGENAKPCCTDPNSG	CCCSSKTNNCCKSDTKD	CCTGTGQGCKCTGCKCCE	52
TtropMTT1	mod 2	---PVKAD	CCGGVNAKPCCTDPNSG	CCCSSKTNNCCKFDTKD	CCTGTGQGCKCTGCKCCQ	52
TtherMTT3	mod 1	ME--KINN	SCCGENTKICCTDLNRQ	CNCACKTDNCCKPETNE	CCTDTLEGCKCVDCKCCK	52
TtherMTT3	mod 2	----SHVT	CCHGVNVKSSCLDPNSG	YQCASKTDNCCKSDTKE	CCTGTQEGCKCTNCQCYK	52
TtherMTT5	mod 1	MD--KIS-	---GESTKICSKTEEKW	CCCPSETQNCCNSDDKQ	CCVGSGEGGIYVCCKCCK	49
TtherMTT5	mod 2	----VQAE	CKCGPNAKYCCIDPNTG	NCCVCKTKFCSKSDSKE	CCPGGSC	41
TpyrMT-2	mod a1	MDKVNNNN	CCCVESTQTCCSGVASG	----------	--------CQCTNCQCCK	27
TpyrMT-2	mod a2	--KTTVSN	CCCAQNQIKCCSSSNEG	----------	--------CKCTNCQCCK	27
TpyrMT-2	mod a3	---DNKTI	CCCSQNNTKCCSNSNEE	----------	--------CKCTNCQCCK	27
TpyrMT-2	mod a4	--TETKSN	CCCSQNKNECCKGKNQG	----------	--------GTCQNCNCCK	27
TtherMTT1	mod 3	---PVQQG	CCCGDKAKACCTDPNSG	CCCSNKANKCCDATSKQ	---------ECCTCQCCK	43
TtropMTT1	mod 3	---PVQAG	CCCGDKAKPCCTDPNSG	CCCSSKTNNCCKA----	----------DTCECCK	37
TtherMTT3	mod 3	---QAQQG	CCCGDKAKACCTDPNSG	CCCSNKANKCCDATSKK	---------ECCVCQCCK	43
TpyrMT-1	mod 2	---PAKSG	CCCGDKAKACCTDPNSG	CCCSSKTNKCCDSTNKT	---------ECKTCECCK	43
TpyrMT-2	mod b	---QVQVG	CCCGDKAKACCTDPNSG	CCCSSKTKKCCDSTEKK	---------QCDTCECCK	43

Figure 1 Members of *Tetrahymena* subfamily 7a MTs are composed of modules.

3.2 Each isolated *T. rostrata* MT is included in one *Tetrahymena* MT subfamily

A previous *Tetrahymena* MT analysis subdivided family 7 (ciliate MTs) into two subfamilies [2]. Now, three new additional *Tetrahymena* MTs have become available, including the two from *T. rostrata* here described. From the alignment of all already reported *Tetrahymena* MTs, we have constructed the phylogenetic tree showed in Fig. 2, and, again, two clear subfamilies are reported (CdMT subfamily or 7a and CuMT subfamily or 7b). The subfamily 7a consists of *T. thermophila* MTT1, MTT3 and MTT5, *T. pyriformis* MT-1 and MT-2, *T. tropicalis* MTT1 and *T. rostrata* MTT1. Subfamily 7b consists of *T. thermophila* MTT2 and MTT4, *T. pigmentosa* MT-2 and *T. rostrata* MTT2 (Fig. 2).

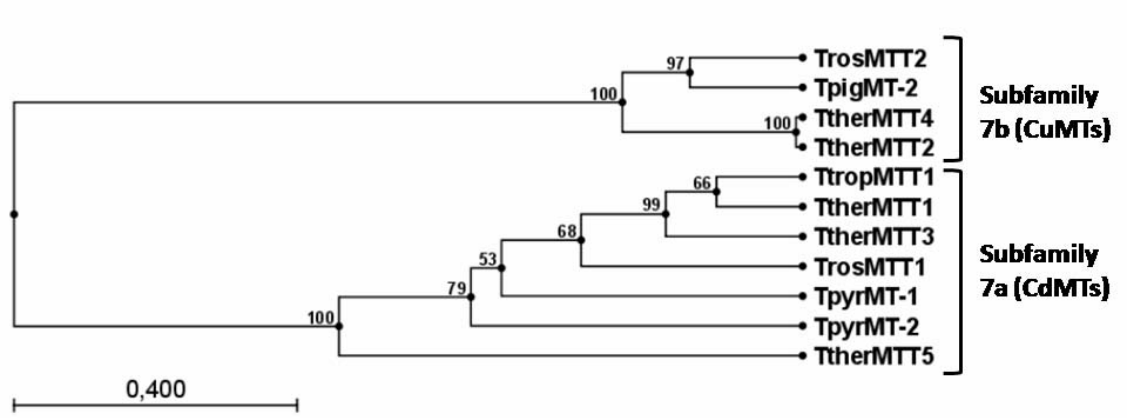

Figure 2 Phylogenetic tree of 11 *Tetrahymena* MTs.

3.3 Expression analysis of *TrMTT1* and *TrMTT2* by real-time quantitative RT-PCR

Gene expression analysis of both *T. rostrata* MT genes were carried out by real time quantitative RT-PCR. In Fig. 3 we show the relative mRNA expression levels of both genes, *TrosMTT1* (Fig. 3A) and *TrosMTT2* (Fig. 3B), induced by various heavy metals (Cd, Cu, Zn, Ni, Pb or As). *TrosMTT1* consistently showed the highest induction levels, relative to α-tubulin (Fig. 3A), with regard *TrosMTT2* (Fig. 3B) for the same heavy metal treatments. Like it occurs in *TtherMTT5* [2], *TrosMTT1* showed a significant induction by Cd and Pb after 24h treatment, lower induction was obtained by Cu, Zn, Ni or As (Fig. 3A). *TrosMTT1* achieved its highest expression level in the presence of Pb (Fig. 3A). MTs have been shown to be induced by lead in rats, humans and fish, also Pb can displace Cd from CdMTs [8]. On the other hand, *TrosMTT2* showed a significant induction by Cu, Zn or Cd, like it occurs in other CuMT (*TpigMT-2*) [9], achieving similar expression values, it is lower induced by Pb or Ni, and down-expressed by As (Fig. 3B). These gene expression levels corroborate the identification of both *T. rostrata* MTs, as Cd- and CuMTs, respectively.

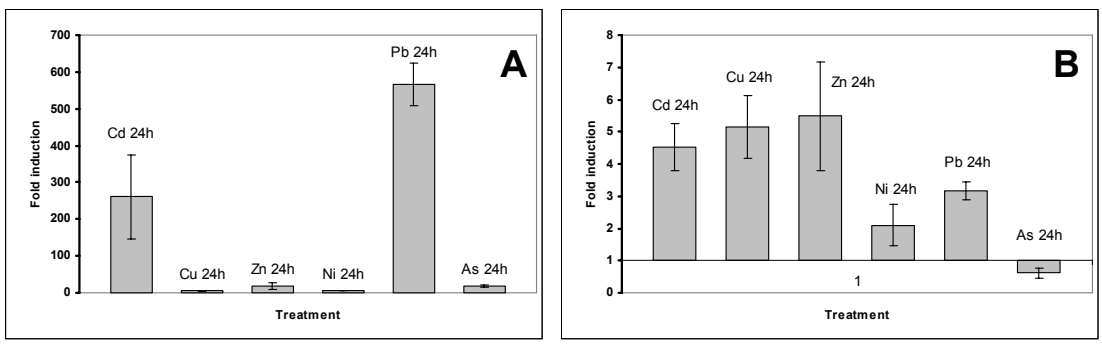

Figure 3 Relative expression levels of *T. rostrata MTT1* and *MTT2* genes obtained by qRT-PCR. (A): *TrosMTT1*, (B): *TrosMTT2*.

3.4 Conclusion / Significance

1)-We have isolated and characterized two new putative MTs from the ciliate *T. rostrata*. They have been identified as Cd- and CuMT, based on their similarities to other Tetrahymena MTs and their relative expression levels to diverse heavy metal treatments. 2)- These two new Tetrahymena MTs corroborate the previous known data and ciliate MT classification. 3)- The gene induction of CdMTs seen to be more specific to certain heavy metals than that of CuMTs. 4)- Two well defined MT subfamilies are established into the ciliate MT family. 5)- TrosMTT1 corroborates the modular /submodular structure of these molecules, and 6)- The theoretical metal binding capacity of these MTs is considerably higher than that reported in standard MTs (metal ions/MT molecule), which might be an important reason to use these proteins as molecular elements for the future elaboration of molecular heavy metal biosensors.

Acknowledgements This research work was supported by a grant from Ministerio de Educación y Ciencia (Spain), project CGL2005-00548/BOS. Ph.D. scholarship from Universidad Complutense (UCM) is awarded to F. A.

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Microbiology Education

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A new strategy for introducing Secondary school students to Microbiology and Biotechnology

J. M. Fernández-Novell[*,1], **D. Cifuentes**[1], **C. Madrid**[2] and **J. C. Ferrer**[1]

[1] Department of Biochemistry and Molecular Biology, [2]Department of Microbiology, University of Barcelona, 08028 Barcelona, Spain

[*]Corresponding author: e-mail: jmfernandeznovell@ub.edu, Phone: +34 93-4021209

We have developed a one-day practical session to present microbiology and biotechnology to science secondary school students. For the last three years, this session has been attended by over 100 students each year, who were selected from High Secondary Schools from all over Catalonia. The feedback that we have received from the students has been extremely positive. Almost all of them described the session as a helpful experience for their preparation and the vast majority appreciated the opportunity to perform practical work in a microbiology laboratory.

Keywords microbiology; biotechnology; secondary school; students.

1. Introduction

In the last 30 years, biotechnology and microbiology have experienced a rapid development. The very large amount of new information gained has many implications, from scientific and economic issues to those related to health or the environment.

Biotechnology and microbiology are not taught as separate subjects in Spanish secondary schools, and very few concepts in biotechnology and microbiology are covered in biology courses [1]. As a result, secondary school students frequently do not appreciate the important role of those disciplines in our every day lives.

For more than ten years the Department of Biochemistry and Molecular Biology at the University of Barcelona has been offering the summer course "I Love Biochemistry", for talented secondary school students [2, 3]. The aim of this course is to bring biochemistry, biotechnology and microbiology close to these advanced students.

Together with the Department of Microbiology at the University of Barcelona we have designed an introductory session specifically oriented to provide them with the opportunity to gain an informed opinion and debate about some key issues in the areas of microbiology and biotechnology.

This introduction allows students to:

- Explore and investigate several uses of enzymes and microbes, for example, in the brewing, baking and dairy industries.
- Isolate and partly characterise microbes from the soil, to appreciate the large diversity of micro organisms present in a given environment.
- Isolate and manipulate DNA and gain a basic understanding of the principles of genetic engineering.

This introductory session is attended by about 100-110 students each year, who are selected from High Secondary Schools from all over Catalonia.

In the session, students are presented in groups of 6-8 students [4] with:

- Several inoculated Petri dishes containing general and selective media.
- Microscopy preparations stained with specific reagents.

They are asked to examine them under a magnifying glass and a microscope. From their observations students are then expected to answer a series of questions.

2.1 Observation of bacterial morphologies

The criteria used for microscopic identification of prokaryotes include cell shape and grouping, Gram-stain reaction, and motility. Gram staining is a common procedure in the traditional bacteriological laboratory [5-7] and allows distinguishing two kinds of bacteria based on their structure. This technique is based on the chemical and physical properties of bacteria cell walls.

Slide preparation procedure: Transfer and spread a drop of the suspended culture or a colony from a Petri dish to be examined on a slide with an inoculation loop (only a small amount of the sample is needed). A flame could be used to facilitate the cell adhesion to the slide.

Gram staining procedure: Add crystal violet stain over the cells on the slide (5-30 seconds). Decant the stain and softly wash the excess stain with running water. Then add enough iodine solution to cover the fixed cells (10-60 seconds). Decant the excess of iodine and wash with running water. Eliminate the excess of water from the slide surface. Then stain with basic fuchsine solution (40-60 seconds). Decant the excess of basic fuchsine solution, wash with water and finally remove the excess of water.

Microscopy observation results: Micro organisms that are stained by crystal violet are classified as Gram-positive and appear in purple. Streptococcus (pneumonia), Staphylococcus (aureus) and Clostridium (tetanomorphum) are some examples of Gram-positive micro organisms. Other micro organisms that are not stained by crystal violet but are stained by basic fuchsine solution are referred to as Gram-negative and appear in pink. Neiseria (meningitides), Enterobacteriaceae (coli), Haemophilus (influenza) and Campylobacter (cholera) are some examples of Gram-negative.

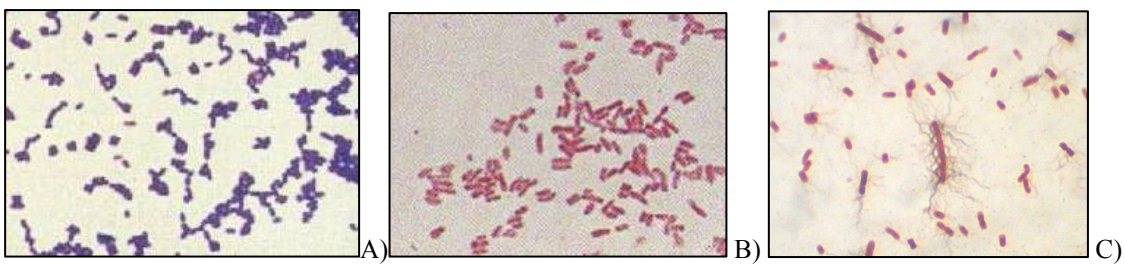


Fig. 1. Fixed bacterial cells stain either as Gram-positive (A, in purple) or Gram-negative (B, in pink). The motility of bacteria is due to the presence of flagella that can be also observed with special staining techniques as seen in C.

TSA (tryptone, soy, agar) is a rich culture medium that permits the general growth of a large range of micro organisms. Since in this kind of medium, each colony corresponds, in general, to an original micro organism, it is adequate for the quantification of the total number of micro organisms in a given sample as in Fig. 2A. For a revision of culture medium see references 8 and 9.

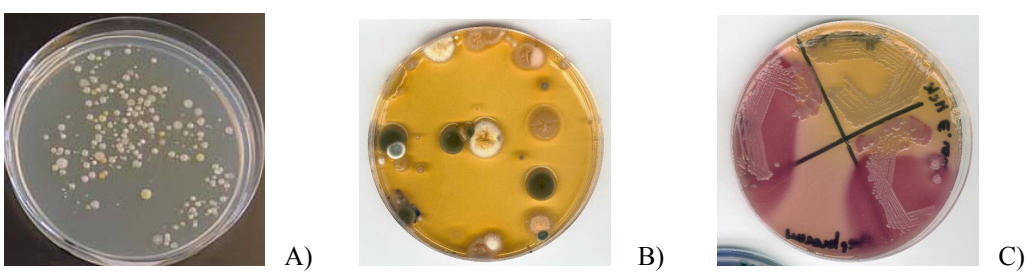


Fig. 2. Petri dishes with A) Tryptone soy agar, B) Saboureaud dextrose agar and C) MacConkey agar.

Saboureaud Dextrose Agar is useful for the isolation and enumeration of fungi (Fig 2B). The low pH of the medium, approximately 5.6, and the high glucose concentration (40 g/ L) favour the growth of fungi, while discouraging that of bacteria.

MacConkey Agar is a selective medium for enter bacteria, since it contains bile salts that inhibit the growth of other bacteria. Additionally, it contains lactose as a carbon source and a pH indicator (neutral red). When a bacterium that can use lactose grows in MacConkey agar, the pH indicator turns red. In the MacConkey Agar plate shown in Fig 2C, one of the bacteria inoculated is not enter bacteria, and therefore is not able to grow in this medium. Two of the other three are able to use lactose, producing a red colour, while the fourth one is unable to metabolise lactose.

In citrate medium, bacteria able to use citrate as a carbon source are easily detected due to the presence of an indicator that turns intense blue upon growth. In the plate shown (Fig 3A), only one of the bacterial strains inoculated can metabolise citrate.

Hektoen enteric agar is another selective and differential culture medium (Fig. 3B) adequate for isolation of pathogenic enter bacteria. In this medium, colonies of Salmonella are dark (blue-green), generally with a black centre, while other enter bacteria produce colonies of salmon colour.

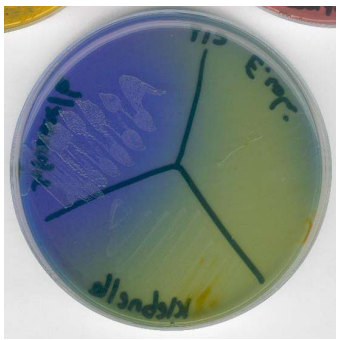
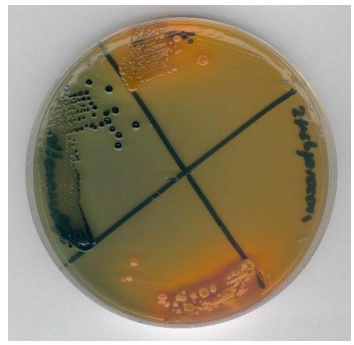
 A)  B)

Fig 3. Petri dishes with A) Citrate medium agar, and B) Hektoen enteric agar

2.2 DNA isolation

DNA was isolated in 1869 by Friedrich Miescher [revision 10]. In 1919 Phoebus Levene identified the nucleotide unit [11]. In 1928 Frederick Griffith suggested that DNA carried genetic information, which was confirmed in 1944 [12]. In 1953 James D. Watson and Francis Crick published the first DNA model in Nature [13, revision 14].

Isolation and purification of DNA is the first step in many molecular biology studies and all recombinant DNA techniques.

The extraction of DNA from biological material requires: cell lysis, inactivation of cellular nucleases, and separation of the desired nucleic acid from cellular debris.

Common lysis procedures include:

- Mechanical disruption.
- Chemical treatment (for example, detergent lysis, chaotropic agents, thiol reduction).
- Enzymatic digestion (proteases).

Cell membrane disruption and inactivation of intracellular nucleases may be combined. For instance, a single solution may contain detergents to make soluble cell membranes and strong chaotropic salts to inactivate intracellular enzymes. After cell lysis and nuclease inactivation, cellular debris may easily be removed by filtration or precipitation.

Purification methods

Some combinations of extraction/precipitation, chromatography, centrifugation, electrophoresis and affinity separation methods are the most often used for purifying nucleic acids as DNA from cell extracts. In the extraction/precipitation methods a mixture of phenol and chloroform is commonly used to eliminate proteins (the main DNA contaminant). DNA is subsequently precipitated and concentrated with ethanol or 1-propanol.

DNA isolation from onion and liver:

- 4-5 pieces of onion or liver were cut and placed into a mortar with some NaCl, some sand and 10 mL of water (50-60ºC)
- The mixture was minced for 2-4 minutes. Then it was filtered using a funnel fitted with woolen glass. The filtrate (6-12 mL) was collected in a test-tube.
- Home cleaning detergent (diluted 1/6 in volume) was added to the filtrate and the mixture was gently shaken for 2-3 minutes and then left to sit for 5-10 minutes.
- An equivalent volume of ethanol 95% at -20ºC was slowly added.
- Two phases formed: the aqueous one in the bottom of the test-tube and the alcoholic one on top. After 3-5 minutes DNA appeared as a white coloured substance in the interphase. It was picked up with a glass wand.
- A small fraction of the DNA was placed into a test tube and dissolved in 4% NaCl. The red coloured solution obtained after adding 5 drops of phenol red confirms the acidic nature of the molecule

3. Session feedback

After the session, the student's opinions were assessed. The feedback that we have received from science secondary school students has been extremely positive. Almost all the students described this session as a helpful experience and useful preparation for higher education in microbiology. The majority appreciated the opportunity to perform practical work in a microbiology laboratory. They stated that these experiments were of great help and they had gained useful knowledge through the experience.

Finally, the experiments and topics discussed and worked with could be addressed in the student's secondary classrooms. On the basis of our experience, we encourage proposals of this kind with the aim of bridging the gap between the secondary school and the university.

Acknowledgments We thank Anna Adrover for her skilful assistance in preparing laboratory materials.

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The Microorganisms in the Portuguese National Curriculum and Primary School Textbooks

Paulo Mafra[*,1,3], **Nelson Lima**[2,3]

[1] Department of Natural Sciences, ESE of Bragança, Campus de Santa Apolónia – Apartado 1101-5301-856 Bragança, Portugal
[2] DCILM, Institute of Child Studies, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal
[3] LIBEC/CIFPEC, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal

[*]Corresponding author: e-mail: pmafra@iol.pt, Phone: +351919239722

The main aim of the present work is the content analysis of the Portuguese National Curriculum and the Primary School textbooks where microorganisms are concerned. The content analysis through categories created *a priori* were used as methodology. In all analysed documents the topic microorganisms did not emerge in a clear way. However, several indirect themes related to microorganisms were found in the National Curriculum and textbooks of the Environment Study issue. These themes can be explored with pupils through experimental activities. The Science Education in primary schools can be introduced with proposals of activities involving microorganisms and contributing to a better understanding of the children's world.

Keywords Textbooks analysis; Microorganisms; Primary School

1. Introduction

From an early age children are told about microorganisms. The concept about microorganisms is ordinarily developed in the children because of the diseases that they or their parents and relatives normal experience. In this way, the concept about these intangible living organisms is naturally coined negatively. Besides this blurred and negative representation it is important that children know the other-side of microorganisms; in general the recognition of the importance of these living organisms to their life and to the planet. It is also important that they discover some of essential and tangible roles that microorganisms play in the world like in production of different kinds of food, in the environment, industry, and in health when helping us fight diseases with antibiotics.

We carry out this research in order to answer the following question: Are the Portuguese National Curriculum and the Primary School textbooks concerned with the plurality of activities of microorganisms? To answer this research question we took into consideration that the textbooks are a didactic resource both to teachers, who use them as a guide in the classroom and to students whose textbooks are, for most of them, the only way of accessing science (Silva, 2001). So, the National Curriculum and textbooks of Primary School became the preferential source to carry out our research, through the content analysis. Using this technique for making inferences by objectively and systematically identifying specified characteristics of messages, we expected to get a better understanding of the contents and concepts that the Portuguese Primary School has about microorganisms. This survey is an essential approach if we want to develop a coherent and applicable action plan to initiate teaching microbiology in the Primary Schools.

2. Methodology

Sixteen textbooks of *Environment Study* of Primary School [M1-16] (from the 1st to the 4th grades), which included 4 publishers for each grade, were selected to be analysed. The National Curriculum of Primary School (Ministry of Education, 2004) was also analysed.

To carry out the analysis of the texts and images of the textbooks *a priori* categories of analysis were defined. In more detail, for the text analysis two categories were defined as presented in Table 1.

Table I – Categories of the analysis of the text in textbooks

Category	Definition	Example
Informative	Text that just means to inform about a content spreading facts and concepts.	"…skin covers our body protecting it from the entrance of germs and impurities …"
Explanatory	Text in which facts are presented but followed by an attempt to explain their meaning.	"…vaccines are very important. Vaccines protect us from diseases…"

The Portuguese textbooks for primary schools are intensively illustrated. In order to get all information that each image contained, they were analysis using the criteria defined by Drouin (1987), Amador and Carneiro (1999) and Palma (2005). Table II presents the three categories used and their definitions.

Table II – Categories of the analysis of the images in textbooks

Category	Definition
Semiology of images	The image can be classified through photography, drawing or scheme.
Pedagogy of images	Know the role of the image in the text to see if its function is to motivate, help the memorization or even if it has an aesthetic, explanatory and metalinguistic function.
Image and conception	Know if the image transmits a visualization of the mentioned concepts. In this case, this category will not be used due to the fact that the content is not explicit in the curriculum and in textbooks. Therefore, conception will always be present in a broader way in the analysis through the study (and not only through the images) in the search of signs which indicate the possibility of exploration of the microorganisms themes in the Primary School.

Finally, in what microorganisms are the contents of the National Curriculum of Primary School concerned and its related documents were analysed taking into account the following four parameters:

1- Microorganisms as a part of the living world (*e.g.* biological diversity, the Kingdoms, classification of living organisms);
2- Microorganisms and health (*e.g.* vaccines, diseases, hygiene of the body, food and places);
3- Microorganisms and food (*e.g.* production; time of shelf-life, food transformation and preservation);
4- Microorganisms in industry, technology and environment (*e.g.* treatment of wastewater and pollution).

3. Results and Discussion

The topic Microorganisms is not directly observed and mentioned in textbooks or in the National Curriculum of Primary School. What it was found is that this topic appears indirectly or implicitly being in some issues associated with the curriculum. Indirectly or implicitly means references in curriculum and textbooks contents which are related to microbial activities. Table III summarised the issues in the National Curriculum of Primary School in which was consider to have any relation with microorganisms.

The microorganism's topic also appears in textbooks indirectly. In this case it is related with health/hygiene, food and environment contents. To illustrate the findings, some examples of contents that we have found in textbooks related with analysis parameters along the four grades will be presented next.

Table III – Issues of the National Curriculum of Primary School where the topic "microorganisms" is indirectly mentioned

Grade	Set of the Curriculum	Thematic
1st	Set 1 – *Knowing yourself* Point 4: *Your body's health*	Identification of the rules of body and food hygiene: wash hands before eating, brush the teeth, wash the food; the knowledge and application of the rules of supervision of his/her health (going periodically to the physician, individual health record)
	Set 3 – *Knowing the natural environment* Point 1 – *The living organisms and their environment.*	Showing animal and vegetable's life (identification of plants and animals and their relating environment).
2nd	Set 1 – *Knowing yourself* Point 4: *Your body's health*	Habits of daily hygiene, importance of potable water, expiry date of food, hygiene of places of collective use; recognition of the importance of vaccination to the health.
	Set 3 – *Knowing the natural environment* Point 1 – *The living organisms and their environment.*	Showing animal and vegetable's life (identification of plants and animals and their relating environment).
3rd	Set 1 – *Knowing yourself* Point 3: *Your body's health* Point 4: *The safety of your body*	Importance of fresh air to health; recognition of some first aid rules.
	Set 3 – *Knowing the natural environment* Point 1 – *The living organisms and their environment.*	Making experiences and observing reproduction of plants, identifying factors of the environment which restrict animals and plants' life. Use criteria to compare and classify plants.
	Set 4 – Knowing the inter-relationship between places. Point 5: *The local trade.* Set 5 – *Knowing materials and objects* Point 4: *Handling objects in concrete situations*	How food is conserved, storage and handling conditions. Expiry date of food. Microscope handling.
	Set 6 – *Knowing the inter-relationship between nature and society.* Point 1: *Agriculture and the environment.* Point 2: *Cattle creation in local environment.* Point 4: *Fishing activity.* Point 8 : *Buildings in local environment.*	Agriculture as source of stocks (wheat/flour, grapes/wine, tomato/tomato paste). Production of dairy products and cured sausage. Canned food. Importance of basic sanitation and water storage for human populations.
4th	Set 1 – *Knowing yourself* Point 1: *Your body's health* Point 2: *The safety of your body*	Identification of skin protective function. Know the basic first-aid rules.
	Set 5 – *Knowing materials and objects* Point 4: *Handling objects in concrete situations*	Microscope handling.
	Set 6 – *Knowing the inter-relationship between nature and society.* Point 1: *Main national productive activities.* Point 2: *The environment quality.*	Identification of products of the Portuguese industry: canned food, etc. Identification of polluting factors, water quality and ways of pollution in water streams, environmental instability forced by human activity.

3.1 Results and discussion by parameter of analysis

3.1.1 Parameter 1 – Microorganisms as a part of the living world

Microorganisms are not referred to as making part of the living world neither in the National Curriculum of Primary School nor in the analysed textbooks. In the syllabus set in which it could be more directly mentioned – *Knowing the natural environment* - we pointed out that the representativeness of the living world is exclusive for animals and plants. Nevertheless, in textbooks M4 to M9 the word "germs" emerges infrequently, however without any association with the living world.

In this parameter of analysis an old scientific concept, which is actually unacceptable, defined mushrooms as plants [M6, 7 and 10]. This confirms the narrow vision given to this topic. The contents related with microorganisms appear in the text in an informative way and the images are shown by photo or drawing with a motivating or metalinguistic function.

3.1.2 Parameter 2 – Microorganisms and health

Under this parameter, the body care emerges as the main topic followed by drawings of children having a bath and brushing their teeth, which predominantly has an explanatory function.

The approach to the vaccines topic usually has an informative nature. However, in M4 we found a picture of a child smiling followed by a text that clearly has a motivating function: "vaccines are very important. Vaccines protect us from diseases". This text has an explanatory function, showing the reason why vaccines are important. Another occurrence was found in M2, in which there is a drawing that shows a child who had been vaccinated playing with a healthy appearance. In contrast, we can observe another child with an ill appearance and who has previously refused to take the vaccines. This picture has a metalinguistic function since no text was found to explain it. In other words, it means that the picture needs to be understood by the students to learn the importance to be vaccinated.

In 4th grade, in the topic concerning the skin function, the word "germ" appears in informative texts. Associated to these texts we found images of a schematic skin with an explanatory function. In this topic we have found an idea that "skin prevents germs from entering in human organism" [M15]. This idea is partly true, however, we did not find complementary information saying that these organisms can enter in our body using other ways.

3.1.3 Parameter 3 – Microorganisms and food

Relatively to food production, it is a poorly mentioned topic in 1st grade textbooks; it only appears in some images with a metalinguistic function. However, in 3rd and 4th grades there are clearer associations among some raw materials and the final products: "…corn is used in bread production…" [M13], "…other products derived from milk like yoghurts, cheese and butter" [M14]. Despite the text being always informative, the existing relation between the raw materials and the transformed product is clear; it is also clear the absence of association between microorganisms as agents of the transformation and the final product. The pictures associated to the text appear as photos with a motivating function, because they do not show the transformation process, they only show the raw material and the final product.

Regarding food expiry date (shelf-life time), we have observed informative texts in some photos of food packaged labels where the date is put in evidence (explanatory function): "people should not eat food after the mentioned date" (M5). We have also found explanatory texts: "food goes off, too. It has an expiry date. After that date, it can cause many problems" [M6].

Food preservation is well shown in 3rd grade textbooks. Sentences like "…in order for food not to go off, several preservation processes have been invented. At home we have frozen food, canned food…" [M10] reflect an explanatory nature because they go further when they explain why food must be preserved.

3.1.4 Parameter 4 – Microorganisms in industry, technology and environment

In 1st grade textbooks, the information related to this thematic is limited to the appearance of the terms "potable water" and "water pollution", without any explanation. In M6, we found a diagram that represents the course of wastewaters from houses to a structure like a WTP (Wastewater Treatment Plant) and this has clearly a metalinguistic function. The picture also shows an alternative concept because the drawing puts the WTP underneath houses, i.e. in the subsoil.

In 2nd grade, we found references to potable water in informative texts, but also with an explanatory nature: "This water is clean, it has not debris nor germs (…) it is drinkable. It is potable water…" [M8]. This is an attempt to defining potable water. This sentence is related with a figure that represents drawings (taps, water bottles) with an explanatory function.

In 3rd and 4th grades, this theme is focalised in pollution causes (cattle creation and some industries polluted the environment). There are informative texts connected with figures like pictures which show discharges of polluted water by industries in rivers which have an explanatory function. In 4th grade textbooks, there is still a relation between pollution and the appearance of the diseases in animals and plants that involving environment. To illustrate this concept, pictures of dead or weak animals in a polluted environment were found near the text and have an explanatory function.

4. Further Information

This study shows that microorganisms are not clearly mentioned in Primary Scholl textbooks or in the National Curriculum. On the other words at this level they are not considered part of the living world. The absence of this topic in National Curriculum of Primary School has the consequence that the theme does not appear structured in textbooks. From a pedagogic point of view it is disturbing that, when microorganisms (germs) are mentioned

in textbooks, it happens in an incomplete or even wrong way leading to possible didactic obstacles for students (Clément, 1998).

Considering that one of the main aims of the Environment Study is to contribute to a better perception and understanding of the child's involving physical-natural environment, we think this omission can potentially generate wrong conceptions about the living world and it is also a narrow perception of the child's involvement in the physical-natural world.

Through this analysis we have seen that in The Environment Study curriculum and in textbooks there are moments in which the theme "microorganisms" can be explored through experimental activities using negative and positive aspects of their activities. Therefore, studies that focus on this topic can contribute to a better understanding of how microbiology can be initiated at this level and motivate the establishment of experimental activities proposals.

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Yeast stress enzymes – application of microbiology and bioinformatics for initiate high school students in environmental studies

I. Alves-Pereira [1,2] and R. Ferreira [*,1,2]

[1] Departamento de Química, Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal
[2] Instituto de Ciências Agrárias Mediterrânicas (ICAM), Apartado 94, 7002-554 Évora, Portugal

[*]Corresponding author: e-mail: raf@uevora.pt, Phone: +351 266745300

The aim of this work was to prepare a summer course for high school students of Portugal which illustrate the importance of microbiology and bioinformatics applications in environmental studies, emphasizing that molecular mechanisms of response, repair and adaptation, endows the cell with essential plasticity to adjust to environmental events, by a process termed stress response. Five high school students with ages ranging from 15 to 17 years old are executes in our laboratory very simple experiments observing that vanadium presence in culture medium, switch on a yeast stress response. This course covers the genomic and functional characterization of CAT T from yeast by bioinformatics search, experimental detection and its response to the vanadium presence in culture medium. The obtained results, namely CAT T detection, its positive response to vanadium and structural and metabolic characterization of gene *CTT 1* products reveal to the students the importance of yeast enzymatic detection as environmental response markers.

Keywords vanadium stress; catalase; *Saccharomyces cerevisiae*

1. Introduction

Ciência nas Férias is a work program supported by Science and Technology Foundation (FCT) of Science and Technology Ministry of Portugal which give an opportunity to the high school student's aproximate to the research scientific work reality, through participation in scientific stages in laboratories of different scientific research institutions which generally occur in summer, during scholar holidays. Bearing in mind that it important stimulate high schools students of Portugal for applications of microbiology and bioinformatics in environmental studies, the Institute of Mediterranean Agrarian Sciences (ICAM) and Department of Chemistry of University of Évora are projected in past July a summer course, which cover the scope of environmental analysis using very simple experiments involving bioinformatics enzymes search, its detection in yeast extracts, as well as, its response to the presence of metal (vanadium-V) in culture medium. The selected theme emphasizes the fact of capacity for adaptation to environmental changes is a universal prerequisite for organism's survival and evolution, enhancing that molecular mechanisms of response, repair and adaptation greatly conserved across nature, endows the cell with its essential plasticity to adjust to environmental events, by a process which is termed stress response. We point up that reduction of dioxygen (O_2) to water via the acceptance of four electrons, during respiration generated reactive oxygen species (ROS) such as the superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$) which are capable of damage DNA, protein and lipid membranes and cause degenerative diseases such as cancer. Moreover, we also call attention to the feature of cells contain antioxidative enzymes such as catalases (CAT) or antioxidants molecules such as glutathione for defence against ROS. In this scope we have explore with the students the importance of catalase T which play an important role in the acquisition of tolerance to oxidative stress in the adaptive response to the H_2O_2 stress [1, 2, 3]. We also highlight the importance of investigate several information about yeast *CTT1* gene sequence and yeast/ human catalase primary structure, physical and chemical properties, using bioinformatics tools, to understand the universal role of catalases, recognizing yeast as a eukaryotic model to evaluate stress response [4, 5, 6, 7, 8, 9]. Also the students have been contact with the possibilities to obtain papers availables on-line which explain molecular mechanisms of catalase response [9, 10, 11]. It was amazing for us reveal to the young students that cell death with morphological and biochemical properties characteristics of apoptosis was first described in unicellular organisms by [12, 13, 14]. Subsequently, we highlight the importance of a lower eukaryotic organism, such as yeast, could be a suitable model to identify components of the basic and evolutionarily ancient stages of apoptosis. In addition, we explain to young students that exploitation of yeast as tools for studying human and animal apoptosis-regulatory proteins has yielded novel insights into cell death mechanisms, creating opportunities for genetic screens [15] or detection of apoptosis misregulation mechanisms which can be associated with cancer, AIDS, autoimmune and neurodegenerative disorders [16]. We also underline the fact that apoptosis is coordinated by a complex network of regulators and effectors, which can be triggered by various toxins or external signals as reactive oxygen species (ROS) or metal pollutants, and internal

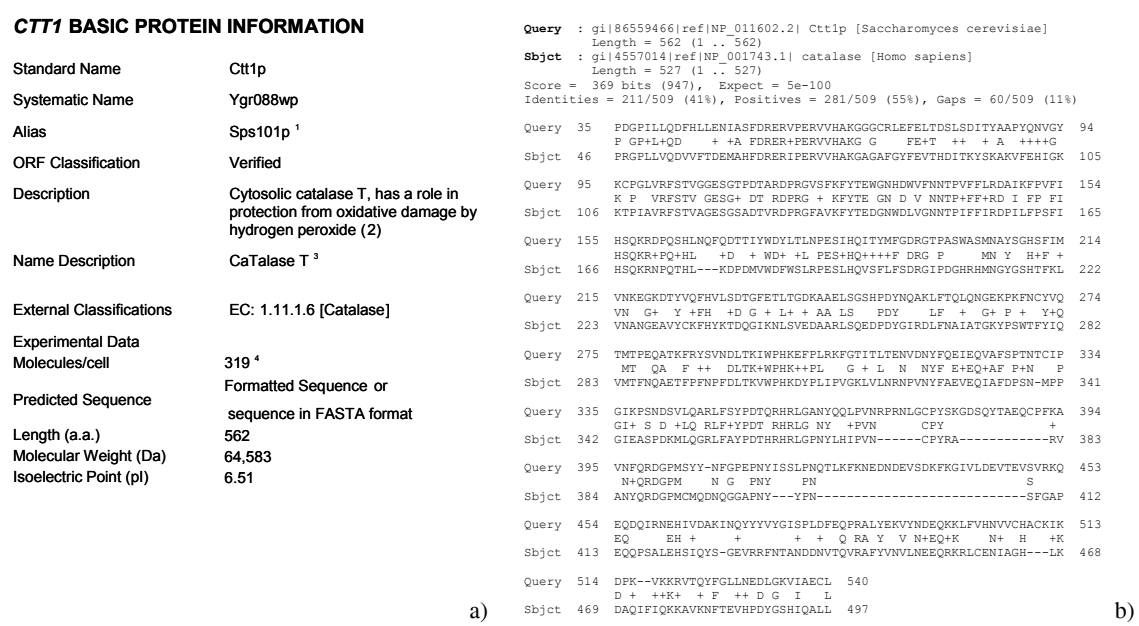
processes as mitotic catastrophe, replication failure or developmentally programmed cell death [17, 18, 19, 20]. Although the stress induction synthesis of cytoplasmic catalase T of yeast *Saccharomyces cerevisiae* occur via the stress response element (STRE) [21, 22], its physiological function suggests that it could also be mediated by ROS being the expression of a catalase gene a facultative anaerobe regulated by stress factors such as heat or metal level in the culture medium.

2. Materials and method

In this work are involved five high school students from the 10th, 11th and 12th class, with ages ranging from 15 to 17 years old, during one week. The students were integrated in experimental activities of our laboratories during summer holidays. A main challenge in this course was to introduce the young students to the internet and to teach them how to effectively use it in research. It was developed an internet assignment that introduces students to websites http://www.yeastgenome.org and http://www.ncbi.nih.gov at beginning of the course. The basic sites introduced cover several subjects, include searching gene *CTT1* sequences of yeast, BlastP of yeast and human peptide sequence of catalase and find its metabolic role. The experimental goals of this learning activity were to execute a laboratory work which involves the culture of wine yeast *S. cerevisiae* vinic UE-ME$_3$, a strain isolated from regional wine of Alentejo, belonging to the Enology laboratory collection of University of Évora, construct its respective growth curve, and evaluate its response in the presence of 25 mM NH_4VO_3, a pentavalent salt of vanadium, using catalase activity as enzymatic marker, having in attention that yeast cells response to stress induced by changes in the environment has been used as a paradigm to study gene regulation. The yeast cells were grown to mid-exponential phase in a water bath, with orbital stirring, at 28ºC, in 250-ml flasks containing 100 ml of YEPD medium with 2% (w/v) of glucose. The cells (10^6 cells ml^{-1}) at mid-exponential phase were inoculated in the same condition in the absence or presence of 25 mM NH_4VO_3, during 200 min. The cells were harvested and ultra-sonic disrupt being the obtained extracts differentially centrifuged at 3000 *g* and 12000 *g* to obtain the post-peroxissomal supernatant, which were used for determination of antioxidant CAT T activity, measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption, according to Beers and Sizer [23]. Protein concentration was determined according to Lowry *et al*. [24] using bovine serum albumin (BSA) as standard. Enzymatic activities were compared by one-way ANOVA ($p<0.01$) [25]. Enzymatic activities and protein contents measurements were carried out in a spectrophotometer, Hitachi-U2001.

3. Results

In order to realise a previous characterization of yeast CAT T, the students used bioinformatics tools to obtained basic information about protein and BlastP Yeast/Human catalase. Fig. 1 shows the previous physical-chemical and metabolic characterization of yeast CAT T and BlastP Yeast/Human results obtained by the students for catalase.



***CTT1* BASIC PROTEIN INFORMATION**

Standard Name	Ctt1p
Systematic Name	Ygr088wp
Alias	Sps101p [1]
ORF Classification	Verified
Description	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide (2)
Name Description	CaTalase T [3]
External Classifications	EC: 1.11.1.6 [Catalase]
Experimental Data	
Molecules/cell	319 [4]
Predicted Sequence	Formatted Sequence or sequence in FASTA format
Length (a.a.)	562
Molecular Weight (Da)	64,583
Isoelectric Point (pI)	6.51

a)

```
Query  : gi|86559466|ref|NP_011602.2| Ctt1p [Saccharomyces cerevisiae]
         Length = 562 (1 .. 562)
Sbjct  : gi|4557014|ref|NP_001743.1| catalase [Homo sapiens]
         Length = 527 (1 .. 527)
Score =  369 bits (947),  Expect = 5e-100
Identities = 211/509 (41%), Positives = 281/509 (55%), Gaps = 60/509 (11%)

Query  35   PDGPILLQDFHLLENIASFDRERVPERVVHAKGGGCRLEFELTDSLSDITYAAPYQNVGY  94
            P GP+L+QD    + +A FDRER+PERVVHAKG G     FE+T  ++  + A  +++G
Sbjct  46   PRGPLLVQDVVFTDEMAHFDRERIPERVVHAKGAGAFGYFEVTHDITKYSKAKVFEHIGK  105

Query  95   KCPGLVRFSTVGGESGTPDTARDPRGVSFKFYTEWGNHDWVFNNTPVFFLRDAIKFPVFI  154
            K P  VRFSTV GESG+ DT RDPRG + KFYTE GN D V NNTP+FF+RD I FP FI
Sbjct  106  KTPIAVRFSTVAGESGSADTVRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDPILFPSFI  165

Query  155  HSQKRDPQSHLNQFQDTTIYWDYLTLNPESIHQITYMFGDRGTPASWASMNAYSGHSFIM  214
            HSQKR+PQ+HL   +D  + WD+ +L PES+HQ++++F DRG P     MN Y  H+F +
Sbjct  166  HSQKRNPQTHL---KDPDMVWDFWSLRPESLHQVSFLFSDRGIPDGHRHMNGYGSHTFKL  222

Query  215  VNKEGKDTYVQFHVLSDTGFETLTGDKAAELSGSHPDYNQAKLFTQLQNGEKPKFNCYVQ  274
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Sbjct  223  VNANGEAVYCKFHYKTDQGIKNLSVEDAARLSQEDPDYGIRDLFNAIATGKYPSWTFYIQ  282

Query  275  TMTPEQATKFRYSVNDLTKIWPHKEFPLRKFGTITLTENVDNYFQEIEQVAFSPTNTCIP  334
             MT  QA  F ++  DLTK+WPHK++PL   G + L  N  NYF E+EQ+AF P+N   P
Sbjct  283  VMTFNQAETFPFNPFDLTKVWPHKDYPLIPVGKLVLNRNPVNYFAEVEQIAFDPSN-MPP  341

Query  335  GIKPSNDSVLQARLFSYPDTQRHRLGANYQQLPVNRPRNLGCPYSKGDSQYTAEQCPFKA  394
            GI+ S D +LQ RLF+YPDT RHRLG NY  +PVN      CPY             +
Sbjct  342  GIEASPDKMLQGRLFAYPDTHRHRLGPNYLHIPVN------CPYRA------------RV  383

Query  395  VNFQRDGPMSYY-NFGPEPNYISSLPNQTLKFKNEDNDEVSDKFKGIVLDEVTEVSVRKQ  453
             N+QRDGPM    N G  PNY    PN                              S
Sbjct  384  ANYQRDGPMCMQDNQGGAPNY---YPN----------------------------SFGAP  412

Query  454  EQDQIRNEHIVDAKINQYYYVYGISPLDFEQPRALYEKVYNDEQKKLFVHNVVCHACKIK  513
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Sbjct  413  EQQPSALEHSIQYS-GEVRRFNTANDDNVTQVRAFYVNVLNEEQRKRLCENIAGH---LK  468

Query  514  DPK--VKKRVTQYFGLLNEDLGKVIAECL  540
            D +  ++K+  + F  ++ D G  I   L
Sbjct  469  DAQIFIQKKAVKNFTEVHPDYGSHIQALL  497
```

b)

Fig. 1 Results obtained by the students using bioinformatics tools on http://www.yeastgenome.org and http://www.ncbi.nih.gov. a) Basic CAT T information. b) BlastP S. cerevisiae/human CAT T peptide sequence.

In the experimental approach young students have acquired basic knowledge about care in preparation of materials and culture media for microbiology and environmental experiments, its importance in the reproducibility of results, as well as, capacity to prepare yeast crude, by ultrasonic disrupt and, understanding its importance for protein and enzymatic activity determination by spectrophotometry.

Consecutively the young students are proceed to obtain *S. cerevisiae* growth curve OD_{640} and CFU counts showed in Fig. 2, and evaluate the response of *S. cerevisiae* to 25 mM NH_4VO_3, using catalase activity as enzymatic marker. Fig. 3 shows the BSA standard curve constructed by the young students for protein determination of post-peroxisomal supernatants. Fig. 4 shows reaction curve of H_2O_2 consumption used to determine the CAT T as well as the effect of 25 mM NH_4VO_3 in yeast catalase.

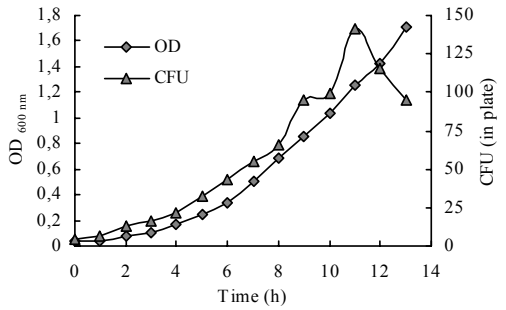

Fig. 2 *Saccharomyces cerevisiae* vinic (UE-ME_3) growth curve obtained growing yeast cells in YEPD medium, at 28ºC and monitoring OD_{640} and CFU

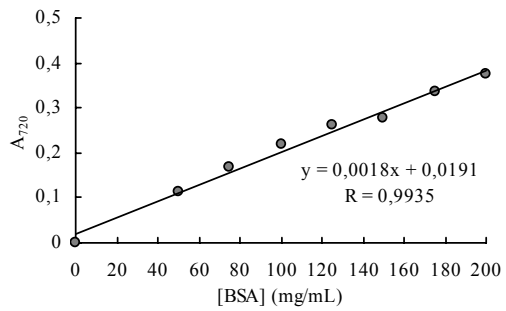

Fig. 3 BSA calibration curve for protein determination in the yeast crude, obtained by ultrasonic disrupt, using Lowry method.

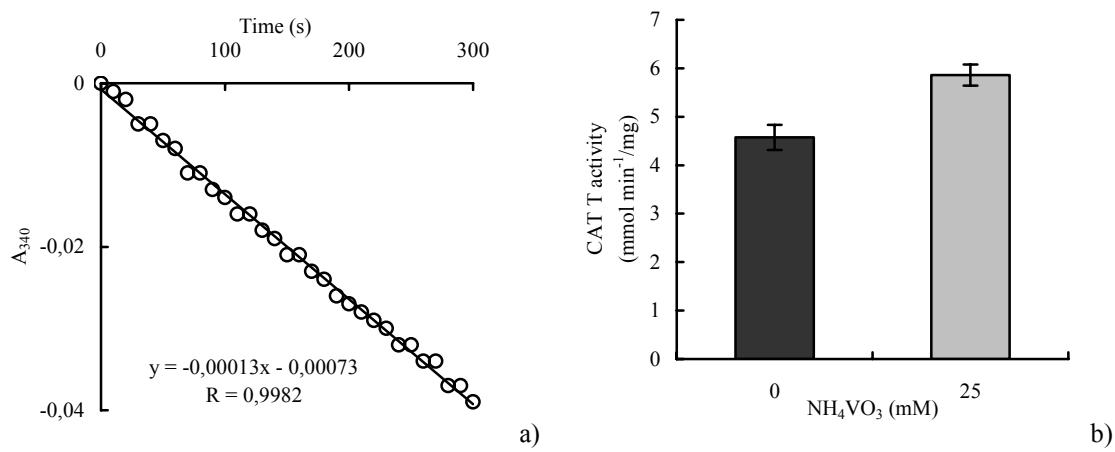

Fig. 4 Antioxidative enzyme CAT T activity of *Saccharomyces cerevisiae* vinic (UE-ME_3). a) Reaction curve obtained in the post-peroxisomal fraction. Cells growing in solid YEPD medium, were harvest, washed and disrupt with appropriate buffer and used for peroxisomal fraction preparation using differential centrifugation (12000*g*). CAT T activity were determined in post-peroxisomal supernatant according [23]. b) Students compare the activity present in the extract of cells growing without NH_4VO_3 or in presence of 25 mM of this vanadium salt.

At last day of the course the students were invited to pronounce their opinion about the course, filling a form which evaluate the theoretical and experimental support of the teachers during the course, the impact of the course on their knowledge about microorganisms research, namely the eukaryotic yeast and its applications in environmental studies. Table 1 shows the main responses of the students about the course.

Table 1 Results from students evaluation of the course.

Students Evaluation		%	Comments
Difficulty	Adequate	100	I like participate in this course:
	Unsuitable		
Timetable	Insufficient		"…by the opportunity to contact with new equipment and substances..."
	Sufficient		
	Good	100	
Duration	Long	20	"…to realize the proposal activities and to obtain experimental results with scientific interest..."
	Adequate	80	
Global evaluation	Wrong		"...by the experiments and persons..."
	Insufficient		
	Suficientt		
	Good	40	"...to execute techniques which determine yeast growth, a long process, and an activity which i'm interest at graduate course and professional level..."
	Excellent	60	
Influence on professional choice	Total according	20	I
	According	80	"...To work in lab activities..."
	Disaccording		
	Total disaccording		

4. Discussion

The obtained results by this class in bioinformatics work, namely higher identity of yeast and human catalases peptide sequences and the crucial metabolic role of this enzyme, are generate in the students a enormous curiosity for the subsequently experimental assays. The positive vanadium response of yeast catalase T, eventually resulting from increase of ROS, can be considered an apoptotic marker. These facts have got illustrates how yeast cells can be useful as eukaryotic model for evaluate the effects of pollutants, particularly metals, on antioxidants systems, suggesting yeast and its antioxidatives systems as potent tools for environmental studies. Also we observed that this course arouse the high school students for an important scientific approach to environmental studies which engage microorganisms as an important piece of the process.

Acknowledgements We thanks to Engº Paulo Laureano of the Laboratory of Enology of University of Èvora for the yielding of the yeast strain. We also thanks to the POCTI Program – Ciência nas férias by financial support of this work at Laboratory of Analytical Biochemistry of Department of Chemistry, SMR-ICAM, University of Évora.

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Bioremediation

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Changes in microbial population affected by physico-chemical conditions of soils contaminated by explosives

B. Limane[1], **J. Juhanson**[2], **J. Truu**[2], **M. Truu**[2], **O. Muter**[*], **A.Versilovskis**[1], **L. Dubova**[1,3], and **Dz. Zarina**[1]

[1] Institute of Microbiology and Biotechnology, University of Latvia, 4 Kronvalda blvd., Riga LV-1586, Latvia
[2] Institute of Molecular and Cell Biology, University of Tartu, 23 Riia Str, Tartu, 51010, Estonia
[3] Latvia University of Agriculture, 2 Liela iela, Jelgava LV-3001, Latvia.

[*]Corresponding author: e-mail: olga.muter@inbox.lv, Phone: +371 67033897

Effect of various amendments added to soils contaminated with explosives was studied under laboratory conditions. A mixture of bacterial isolates was inoculated into soils samples and incubated at +28 °C. After 14 days incubation, the pH and Eh level in the liquid fraction of soils amended with water was 6.73 and +34mV, in turn, with buffered solution was 7.2 and -30 mV, correspondingly. The total count of microorganisms in the samples was dependent on the presence of bacterial inoculum and nutrient amendments. Inoculation of soil samples with mixture of bacterial isolates had a strong effect on microbial community composition revealed by 16S rDNA-DGGE analysis. Several bacterial strains present in inoculum became dominant in TNT and RDX amended samples.

Keywords explosives, soil remediation, microbial community, physico-chemical properties of soil

1. Introduction

Soil contamination with explosives is a serious environmental problem. Various technological approaches for remediation of soils contaminated with explosives have been suggested during last decades. For example, *in situ* technologies based on application of mixture of microorganisms with explosives-degrading ability and amendments. Besides, *ex situ* technologies, such as composting and slurry reactors have been used [1-3]. Each approach has own specificity. However, inoculation with active microorganisms and delivering amendments into contaminated soil is common for technologies mentioned above. The effect of amendments on the change of microbial community in soil during remediation process is known to be one of the most important factors finally influencing the outcome of remediation. Our previous study was conducted with soils from the military camp and was focused on isolation of microorganisms with explosives-degrading ability. The results demonstrated an enhanced ability of bacteria to degrade nitroaromatic compounds in the medium amended with cabbage leaf extract [4-5]. In this work, the impact of microbial biomass addition and various amendments on changes in microbial community of contaminated soils was studied. A slurry-type model was used to provide mass transfer of nutrients and electron acceptors by using mechanical mixing and aeration [3].

2. Materials and methods

Contaminated soil was sampled at the military polygon, prepared as average sample and analyzed for identification of explosives by HPLC according to EPA method [6]. Each sample contained 50 g soil and 30 ml liquid phase. The samples were incubated at +28 °C during 14 days in the dark, periodically shaking. Liquid phase, according to the scheme of experiment, consisted of sterile distilled water, or M8* solution. Cabbage leaf extract, glucose, as well as inoculum of bacteria were added when indicated. M8* solution contained, g/l: Na_2HPO_4 – 60, KH_2PO_4 – 30, NaCl – 5 (pH 6.9). Cabbage leaf extract was prepared from white cabbage leafs. 500 g leafs were washed with tap water, boiled at 100 °C for 30 minutes, cooled, afterwards the liquid fraction of extract was filtered with 0.45 μm filter (Millipore) and steamed for 15 min. The prepared extract was stored at +4 °C until used. Sterility test by plating method was done if necessary. Bacteria associations were originally isolated from explosive-contaminated soils on the basis of its ability to grow with TNT and other nitroaromatic compounds as the sole nitrogen source. The A43 association alone, as well as a mixture of 20 associations (A-Mix) were used. Concentration of inoculum for A-43 and A-Mix was 1.6 x 10^6 CFU/ml. The total microbial count was determined using TGA medium (Sifin, Germany). All chemicals used were reagent or analytical grade. Soil grading composition and chemical content were determined according to [7,8]. Experiments were performed at least in triplicate, within one experiment each variant was performed in duplicate.

Microbial DNA was extracted from soil samples with UltraClean Soil DNA kit (Mo Bio Laboratories, Inc.). Bacterial community structure was assessed with 16S rDNA sequence specific primer pair p338f-GC and p518r [9,10]. A denaturing gradient gel electrophoresis (DGGE) system DCode (Bio Rad, Inc.) was used to separate the amplified gene fragments. DGGE gels were digitized and banding pattern analyzed using cluster analysis based on Pearson correlation coefficient.

3. Results and discussion

3.1 Characteristics of soil

Type of soil was determined as medium coarse sandy soil. Soil pH was 6.3 (in H_2O) and 4.17 (in 1M KCl), Eh was 50 mV (in H_2O) and 131 mV (in 1M KCl). Soil moisture content was 4 %, humus content – 14.72 %, C_{org} – 8.55 %. Carbonates were not detected in given soil samples.

Soil physically-chemical parameters as well as microbial counts were measured separately for two fractions, i.e. thick and liquid. Dry weight of thick fraction among samples varied from 90.61% to 93.92 %, of liquid fraction – from 1.03 to 9.00 %.

Average concentration of explosives in soil before experiment was found to be 150 mg/kg air dried soil. The spectrum of nitroaromatics consisted, in decreasing order, of 2-amino-6-nitrotoluene, 2,4,6-trinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,3-dinitrotoluene, 1,2-dinitrotoluene, nitrobenzene, 2-amino-4,6-dinitrotoluene, and 2,6-dinitrotoluene. Nitramines were not detected in soil samples used in this experiment. The results of the changes in explosives concentrations during experiment indicated t more rapid degradation of nitroaromatics in the samples with buffered liquid fraction. Thus, after 14 days incubation, nitroaromatic compounds were not detected in the liquid fraction of buffered samples, in turn, a liquid fraction of the sample No.1, i.e. with water, contained 21.57 mg/kg air dried soil.

3.2 Changes of pH and Eh in soil samples amended with different components

Measurement of pH and Eh levels revealed the differences of physically-chemical properties between thick and liquid fractions in all studied samples after 14 days incubation. Thus, redox potential in thick fraction was considerably lower as compared to liquid fraction. Besides, this difference was the most expressive in the sample No.1, i.e. with distilled water and achieved -46.05 ± 1.20 mV and 33.50 ± 0.71 mV, correspondingly. Addition of buffered solution, i.e. M8* solution, to soil resulted in decrease of the Eh level in liquid fraction after incubation period (Fig.1a). In turn, the pH level in thick fraction was higher compared to liquid fraction, and this difference was more pronounced in the sample No.1 (with water) (Fig. 1, b). Additional experiments were conducted to clarify whether salt concentration or salt composition plays a role in the changes of pH and redox potential in studied soil samples. Addition of M8* salt solution in concentration diapason from 0.1 % to 1.90 % noticeably changed redox potential without pH changes due to its buffering capacity. In turn, addition of NaCl alone resulted in an increased redox potential and decreased pH level (results not shown).

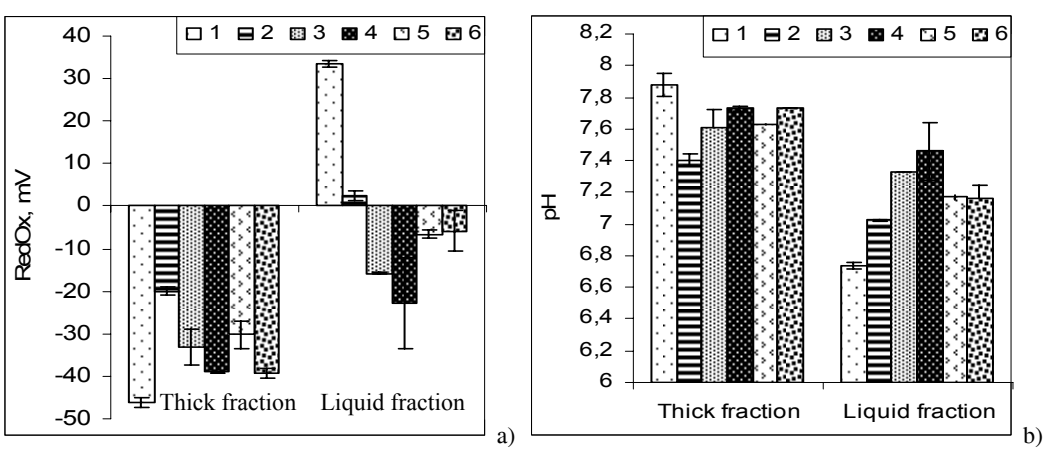

Fig. 1. Effect of various amendments onredox potential (a) and pH (b) of soil samples incubated during 14 days at +28 °C. 1 – soil + dist. water; 2 – soil + M8*; 3 – soil + M8* + A43; 4 – soil + M8* + 1.25% CLE + 0.25% glucose + A43; 5 – soil + M8* + A-Mix; 6 - soil + M8* + 1.25% CLE + 0.25% glucose + A-Mix. Concentration of inoculum for A-43 and A-Mix was 1.6 x 10^6 CFU/ml.

3.3 Changes of microbial community in contaminated soils during incubation with various amendments

As was shown in Fig.1, an amendment of soil with buffered solution resulted in the changes of soil physico-chemical properties, i.e. a decrease of redox potential. In general, the degradation of TNT proceeds more completely and more quickly as the redox potential decreases [11,12]. Besides, these changes could influence microbial activity. Indeed, an increase of the total microbial counts was observed in the soil samples amended with M8* salt solution compared to the samples amended with water (Fig.2).

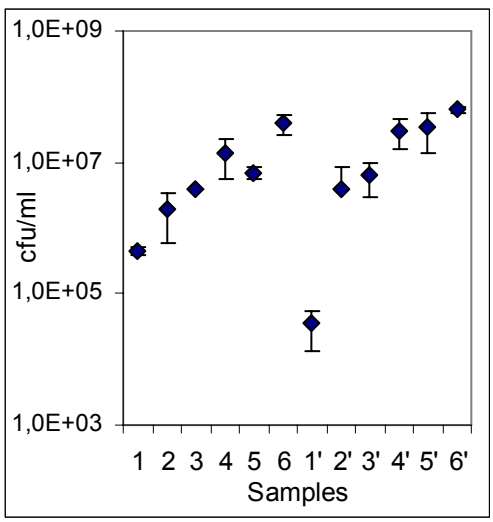

Fig. 2 Effect of various amendments on microbial count in soil samples incubated during 14 days at +28 °C
1 – soil + dist. water;
2 – soil + M8*;
3 – soil + M8* + A43;
4 – soil + M8* + 1.25% CLE + 0.25% glucose + A43;
5 – soil + M8* + A-Mix;
6 - soil + M8* + 1.25% CLE + 0.25% glucose + A-Mix.
Concentration of inoculum for A-43 and A-Mix was 1.6 x 10^6 CFU/ml.

This effect was more pronounced in the liquid fraction of the samples. Addition of cabbage leaf extract and carbohydrates slightly increased the total microbial count after 14 days incubation. Addition of glucose or sucrose gave the similar results in the changes of microorgansims concentration in soil samples (results not shown). Additional testing of soil microorganisms by their cultivation on the selective medium, i.e. containing 200mgTNT/l, was performed. This testing showed, that the samples amended with M8*, carbohydrates, cabbage leaf extract and bacterial mixture A-Mix, contained the highest number of bacteria with TNT-degrading ability, which corresponded to the total microbial number tested on TGA medium. Meanwhile, a presence of culturable bacteria with TNT-degrading ability was not detected in the soil samples amended with water.

Results of 16S rRNA gene based DGGE fingerprints of soil samples show the impact of amendments and bacterial biomass addition on the contaminated soil microbial community structure (Fig.3). According to the dendrogram based on the DGGE fingerprints, microbial communities in soil samples with added bacterial biomass differ from the rest of the samples and form a distinct group. Dendrogram demonstrates that in addition to the effect of bacterial biomass addition, influence of the amendment type to the microbial community structure was also important. Cabbage leaf extract leads to dominance of certain bacterial strains from added biomass, while addition of TNT and RDX favors growth of most bacterial strains from added bacterial mixture.

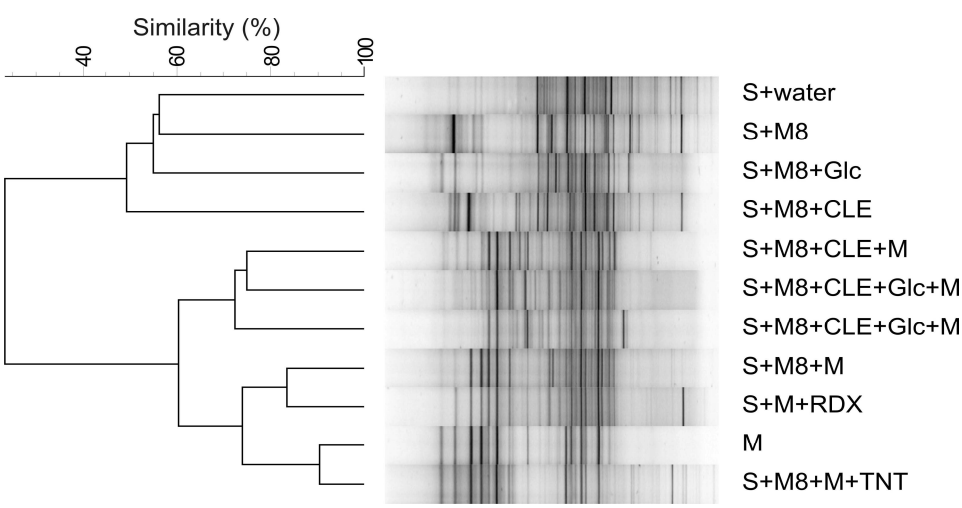

Fig. 3. Dendrogram of soil samples based on cluster analysis of the DGGE profiles of microbial communities. Abbreviations: S – soil, M8 –M8* salt solution, Glc – glucose, CLE – cabbage leaf extract, M – mixture of strains A-Mix.

4. Conclusions

Addition of buffered salt solution to the soils contaminated with nitroaromatic compounds, resulted in decrease of redox potential, which is known to play an important role in the degradation of explosives. Total microbial count considerably increased in the samples amended with buffered salt solution compared to water. Other tested amendments, i.e. carbohydrates and cabbage leaf extract, as well as a mixture of bacteria with explosives-degrading ability, also resulted in an increased total microbial count. Inoculation of soil samples with mixture of bacterial isolates had a strong effect on microbial community composition revealed by 16s rDNA-DGGE analysis. Several bacterial strains present in inoculum became dominant in TNT and RDX amended samples.

Acknowledgements The support by The Ministry of Defence, the Republic of Latvia is gratefully acknowledged. This work was partially financed by Scientific Council of Latvia, Project 933. Collaboration was financially supported by the Estonian Academy of Sciences and University of Tartu.

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DNA and cDNA fingerprinting of 16S rRNA gene to assess the key organisms in low temperature methanogenic consortium

F. A. Chinalia[1,*], R. M. McKeown[1], T. Mahony[1] and V. O'Flaherty[1]

[1] Microbial Ecology Laboratory, Department of Microbiology and Environmental Change Institute, National University of Ireland, Galway, University Road, Galway, Ireland

[*]Corresponding author: email: chinalia@hotmail.com

Low-temperature (<20°C) anaerobic digestion is an emerging, cost-efficient technology, which has been successfully applied to a variety of high-strength hazardous and non-hazardous waste-streams. Operated at low-temperature, expanded granular sludge bed (EGSB) reactors favour the development of a methanogenic consortium adapted to high biodegradation rates. Reactor operating parameters, such as wastewater characteristics and organic loading rate, affect microbial consortium physiology and therefore could shift bacterial 16S rRNA ratios, for example by changing growth rates and/or physiological activity. Thus, as a screening tool, temporal DGGE-profiling, comparing DNA and cDNA, was employed to highlight the potentially key organisms directly involved in psychrophilic (12°C) toluene methanogenesis in a laboratory-scale EGSB bioreactor. Biomass samples were taken and analysed over a 5-day period, immediately following an imposed increase in toluene loading rate. Functionally active potential toluene-degraders were successfully characterized. The results suggested that a putatively psychrophilic *Geobacter*-like organism was involved in the process along with hydrogenotrophic *Methanospirillum*-like and acetotrophic *Methanosaeta*-like *Archaea*. The presence of additional mesophilic and thermophilic putative toluene-degraders suggested, however, that reactor operating temperature may not have been the main factor for the development of this well-established and active microbial methanogenic consortium.

Keywords anaerobic digestion, toluene, 16S rRNA, EGSB reactor, DGGE, *Geobacter*

1. Introduction

Anaerobic digestion (AD) is frequently employed for treatment of various industrial wastewaters, at temperatures ranging from 30-40°C. Despite this, many wastewaters are released for treatment at low-ambient temperature. Thus, anaerobic digestion operated under psychrophilic conditions can present a more attractive and cost-effective treatment option (1). Lowering the operational temperature, e.g. to between 9-15°C, may result in an undesirable decrease in the maximum specific substrate utilization rates. In order to favour high biodegradation rates under psychrophilic conditions, a commonly employed technical approach is the promotion of enhanced substrate mass transfer (1, 2). Expanded granular sludge bed (EGSB) reactor types have been used for this purpose (3). Despite the successful application of low-temperature anaerobic treatment at laboratory-scale, few psychrophilic methanogenic and acetogenic organisms have been isolated to date. In fact, Rebac et al (4) reported that mesophilic organisms may be capable of sustaining low temperature anaerobic treatment, and, consequently, mesophilic biomass-inoculated EGSB reactors may be feasible for full-scale psychrophilic operation, following an adaptation period. This information illustrates not only the lack of knowledge regarding the microbial populations responsible for treating wastewater at low temperatures, but also highlights the unveiled potential of the organisms involved in low-temperature anaerobic treatments. For instance, toluene biodegradation occurs under all of the major anaerobic electron-accepting conditions, but specifically in methanogenic environments where mineralization is often achieved through syntrophic relationships between species within the consortium (5). However, the biochemical nature of such syntrophic relationships is still the subject of speculation, as is the identity of the microbial species involved. The aim of this work was, by combining DNA and cDNA-fingerprinting of the 16S rRNA gene, to identify potentially key organisms involved in toluene biodegradation at low-temperature (12°C) in an EGSB reactor operated under methanogenic conditions. We characterized temporal microbial shifts within the reactor, over a 5 day period following an increase in the toluene loading rate (from 8.7 to 17.8 g m^{-3} d^{-1}). The reactor was operated for 240 days at 12°C prior to the applied perturbation.

2. Material and Methods

2.1 Reactor design and operation

A 3.75l laboratory-scale, EGSB-anaerobic filter bioreactor (6) was operated in a long-term trial (>300 days). The bioreactor was initially inoculated with a 1:4 mixture (134 g of volatile suspended solids (VSS)) of low-temperature granular sludge treating aliphatic solvents (9-15^{o}C) and a mesophilic granular sludge treating dairy-processing wastewater, respectively. The bioreactor was fed with a synthetic wastewater containing acetate, propionate, ethanol and butyrate to a total of 6.75 g chemical oxygen demand (COD) l^{-1} (Fig. 1). The influent was buffered with $NaHCO_3$ (pH 6.8-7.9) and nutrients were supplemented as described previously (6). Organic loading ratios and hydraulic retention times are displayed in Figure 1. The effluent from the reactor was re-circulated at a rate of 5 m h^{-1} and the temperature maintained at 12^{o}C. The influent was amended with toluene at different concentrations during the trial (Fig. 1). For the microbial analysis, the trial was divided into 3 operational phases. The first phase was the 5 day period prior to the increase in toluene loading ratios from 8.7 to 17.8 g m^{-3} d^{-1}, while phase two represented the 5 days of biofilm sampling, followed by 5 days of monitoring the performance (phase three) to verify the steady-state stability based on COD and toluene removal. Biofilm samples for the molecular analysis were obtained on days 220 to 224 (Fig. 1).

2.2 Chemical and molecular analysis

Reactor performance was assessed by COD removal efficiency, methane yield (APHA, 1992) and toluene biodegradation according to Enright et al. (7). Biomass was collected from the bioreactor via a sample port located at the bottom of the sludge bed at intervals of approximately 24 hours, on days 220 to 226, and stored at -20^{o}C prior to testing. Biofilm was crushed (pestle/mortar), centrifuged and 0.5 g was used for DNA/RNA extraction. Extraction and DGGE-profiling was carried out according to Gusmao et al. (8). Bacterial and archaeal DNA and cDNA DGGE profiling were assessed using primers and conditions described by Gusmao et al. (8) and Kudo et al. (9), respectively, and the DGGE protocol described by Muyzer et al (10). Reverse transcriptase PCR of RNA samples was carried out according to Corgie et al. (11). DGGE bands were excised, cloned and sequenced using TOPO kit (Invitrogen), following the manufacturers' specification. Clone libraries were obtained using primers and conditions described by So and Young (12). Clone libraries were screened for distinct operative taxonomic units (OTUs) by digesting 600 clones (300 at day 220 and 225, respectively) with Hae *III* DNAse. Sequencing confirmed 26 distinct OTUs and a phylogenetic tree was constructed using PAUP and Kimura 2-parameter for the analysis of 16S DNA of about 900 base pairs per OTUs.

3. Results and Discussion

A noticeable decrease in COD and toluene removal efficiencies was observed within the 5 days succeeding the increase in toluene concentration from 8.7 to 17.8 g m^{-3} d^{-1} (Fig. 1; day 220). The graph also shows a recovery

A

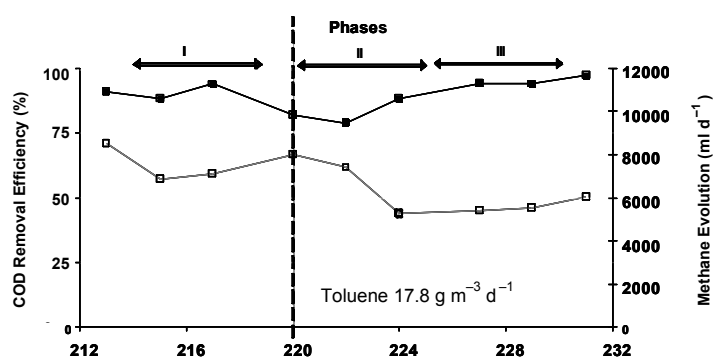

Figure 1: (A) COD removal efficiency (■) and biogas production (□) in the EGSB-AF hybrid reactor treating toluene. (B) Average values of hydraulic retention time (HRT), chemical oxygen demand loading and removal ratios (COD), methane yields and toluene removal ratios.

B

Days	HRT (h)	COD Loading (kg m^{-3} d^{-1})	COD Removal (%)	Methane Yield (ml CH_4 d^{-1})	Toluene Loading (gm^{-3} d^{-1})	Toluene Removal (%)
213-219	24	6.75	91 (±3.00)	7511 (±892.81)	8.7	83 (±2.52)
220-226	24	6.75	83 (±4.58)	6887 (±1430.63)	17.8	69 (±1.53)
227-233	24	6.75	95 (±1.69)	5643 (±340.28)	17.8	90 (±4.73)

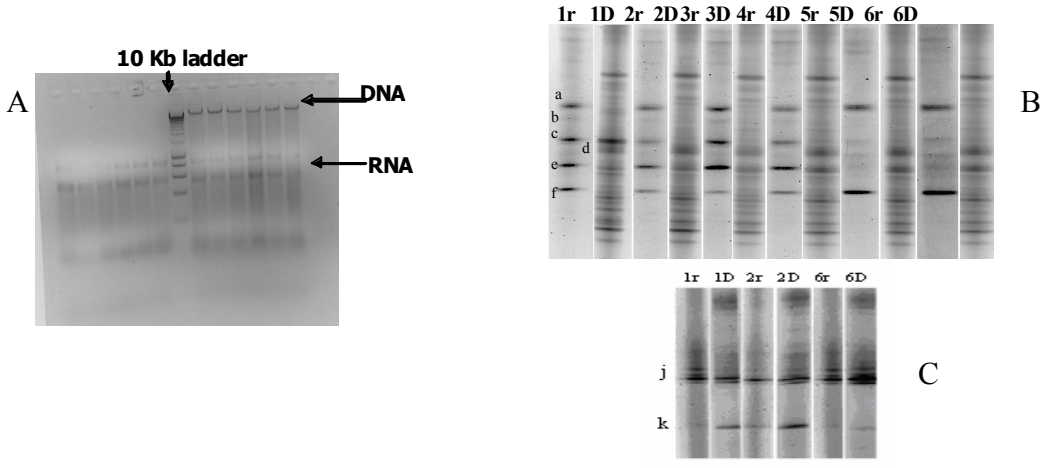

Figure 2: (A) Agarose gel showing DNA and RNA extracted from the samples. (B) DGGE gel showing bacterial DNA (1D to 6D) and bacterial cDNA (1r to 6r) profile characterizing the five consecutive days after toluene amendment of 20 g m^{-3} d^{-1}. Samples were taken in 24 h intervals. (C) Archaeal profiles are illustrated on DGGE gel C showing days 0, 1 and 5 after toluene amendment to 17.8 g m^{-3} d^{-1}. The identified bands were extracted, cloned and sequenced. Bacteroidetes and proteobacteria-like sequences matched most of bacterial sequences and archaeal sequences suggested *Methanospirillum* (j) and *Methanosaeta*-like organisms (k).

in COD removal efficiency and such result is assumed to be the classic period of biomass acclimation before reaching a new steady state of toluene removal ratios.

It was assumed that the presence of 16S rRNAs of toluene-degrading organisms may be found in comparably higher proportion within the total RNA extracts, as a result of stimulated growth and/or physiological activity after toluene amendment. Figure 2 A shows the result of DNA and RNA extraction and Figure 2 B and C the respective DGGE-profiling of cDNA and DNA of bacterial and archaeal 16S rRNA gene during 5 days succeeding toluene amendment.

The DGGE-profiling suggested that neither bacterial nor archaeal diversity changed significantly as a result of toluene amendment. Table 1 showed a clear shift in frequencies of the bacterial groups observed in the clone libraries obtained before and 5 days after the increase in toluene. The groups with significant differences were Bacteroidales- and Proteobacteria- like species. On the other hand, the variation of cDNA band pattern observed in Figure 2 suggested that four bacterial species, out of a potential 6, were comparatively affected during this period. The DGGE bands were compared to the sequences of the clone library. Through this approach, sequences were identified as *Bacteroidetes*-like species (bands a and e; Fig. 2), sequences of proteobacteria (bands d and f), cloriflexi (band b) and unclassified clostridia-like species (band c), respectively. Apart from *cloriflexi* (green non-sulphur bacteria), all the other cited groups may exhibit recognized species of hydrocarbon degraders.

DGGE cDNA band pattern variation suggest that species represented by bands a and f may have played an important role in the process after toluene amendment. These sequences were compared to a clone library produced in this work (about 26 species in 600 clones) and in further analysis using 900 base pairs they matched (≥96% of about 900 bp) the sequences of *Bacteroidete* sp. and *Geobacter* sp. deposited in the ribosomal data base project (RDB) with accession numbers AY548787 and AF5291129. The DNA sequence of the observed *Geobacter* sp. characterizes a psychrophilic species with a recognized potential for anaerobic toluene biodegradation in the methanogenic consortium (13, 5). On the other hand, the clone library also reveals sequences of bacterial species with high degree of similarity with commonly known meso- and thermophilic species, such as *Clostridium thermocellum* and *Thermoanaerobacterium* sp. These conflicting observations regarding physiological adaptation to temperature indicate that the microbial community may be composed of not yet well characterized psychrophilic organisms or both physiological attributes may be present within the members of such biofilm.

It is possible that the source of inocula and operating parameters of the EGSB-AF hybrid reactor have selected and favoured distinct organisms regarding their affinity to temperature. Thus, temperature may have not been the only factor affecting the selection of physiologically adapted organisms. The high mass transference ratios achieved within this reactor type may have also contributed to a high treatment performance without a fully adapted psychrophilic community. It is reported that few psychrophilic methanogenic and acetogenic (syntrophic) organisms have been identified so far (1, 4). Therefore, there is very limited information about quantitative and qualitative characteristics of such microbial consortia. This work suggests that DNA and

RNA-based molecular analyses are important tools for unveiling structural and functional diversity of such microbial communities, particularly when associated with operational parameters. Therefore, it is also possible to suggest that the physiology of non-psychrophilic organisms may have been favoured in low temperature as a result of a balance between microbial biomass of non-psychrophilic organisms and high rates of substrate mass transfer which is often achieved in this type of reactor (1, 4 and 6).

Bacterial Groups	Number of species	% of 300 clones (day 0)	% of 300 clones (day 5)
Clostridiaceae	5	30	28
Syntrophomonadaceae	9	33	29
Chloroflexi -like	1	2	3
Actinobacteria -like	1	5	6
Bacteroidales	6	19	13
Proteobacteria	3	10	21
Spirochaeta	1	1	1

Table 1 Frequency of 26 identified bacterial clones divided in distinct bacterial groups according to the evolutionary distance proposed by the Ribosomal Database Project (RDB). Two distinct clone libraries were assessed before and five days after toluene amendment with 17.8 g m^{-3} d^{-1}.

It is common knowledge that the toluene biodegradation pathway and potential bacterial degrading organisms in methanogenic environments have not yet been fully explored so far. It is suggested that this process is achieved by a syntrophic association between fermenting bacteria and hydrogenotrophic archaea (5). Although there is not an apparent correlation between distinct organisms, our observation suggests that toluene biodegradation may have been a product of a syntrophic interaction between *Geobacter*-like and *Methanospirillum*-like species. The dominant proportion of such organism's sequences in the final cDNA DGGE-profile obtained from the total RNA extracts after toluene amendment, may be a significant indicator that they are key organisms in this methanogenic consortium. *Geobacter*-like species have been previously reported to be directly associated with aromatic hydrocarbon biodegradation (5, 14). The first steps in anaerobic toluene oxidation by *Geobacter*-like organisms require a negative Gibbs free energy which may be provided by hydrogenotrophic organisms such as *Methanospirillum* sp. (5, 15). In this anaerobic consortium, it is believed that original toluene molecules are totally mineralized into CO_2 and CH_4. Therefore, our results show competitive advantageous kinetic rates favouring the treatment of toluene contaminated wastewater under psychrophilic conditions and in the presence of alternative carbon sources if compared with previous literature (7, 16).

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Effect of Dibenzothiophene on the Growth, on the Morphology and on the Ultrastructure of *Cunninghamella elegans*

Patrícia Mendes de Souza[*,1,3]; Petrusk Homero Marinho[2,3]; Marcos Antônio Barbosa Lima[3]; Aline Elesbão do Nascimento[3]; Galba Maria de Campos –Takaki[*,3]

[1] Post-Graduates programme in Biology and Funghi - Federal University of Pernambuco, Recife, PE Brazil;
[2] Post-Graduates programme in Biological Sciences - Federal University of Pernambuco, Recife, PE Brazil;
[3] Nucleus Research in Environmental Sciences, Catholic University de Pernambuco, Recife, PE Brazil; Rua Nunes Machado, 42, Bloco J, Boa Vista, CEP 50050-590, Recife, PE, Brazil

[*]Corresponding authors: e-mail (galba_takaki@yahoo.com.br/tyttams@hotmail.com)

Combustion of petroleum fuels leads to the atmospheric emission of sulfur oxides which are the major cause of acid rain. The growth, the morphology and the ultrastructure of *Cunninghamella elegans (UCP 596)*, were evaluated by scanning electron microscopy, light microscopy and fluorescence microscopy to characterize the metabolic responses of the fungus in presence of DBT. The ability of *C. elegans* to adapt to adverse environmental conditions by changes in its activities physiological, biochemical and morphological aspects, represent key elements in the understanding of cell behavior for the identification of specific mechanisms of development, maturation, differentiation and survival of the microorganism in response to environmental challenges.

Keywords *Cunninghamella elegans*, Dibenzothiophene, Oxidation, Morphology, Ultrastructure.

1. Introduction

Fossil fuels and crude oil contain significant amounts of organosulfur compounds, such as alkyl- and cycloalkyl thiols, and aromatic heterocyclic such as thiophene, which releases sulfur dioxide into the atmosphere during its combustion and causes acid rain and air pollution [1, 2, 3, and 4]. Dibenzothiophene (DBT) is considered a model polycyclic sulfur compound in fossil fuels [5, 6, 7]. It has been reported that some bacteria can use DBT as a sole source of sulfur without breaking its carbon-carbon backbone by using a sulfur-selective oxidative pathway [7, 8]. Many strains of bacteria are not useful for the biosulfurization because they mineralize the carbon skeleton [9]. However, DBT has been widely used as a model compound to screen microorganisms which might be used in desulfurization of fossil fuels [9, 10, 11]. Among eukaryotic organisms, *Cunninghamella elegans* has been reported to desulfurization DBT [12]. This fungus grows on DBT and produces DBT-5-oxide and DBT-5-dioxide but not biphenyl. However, the physiological aspects, such as morphology and ultrastructure response to DBT presence are not reported. Considering the ability of *C. elegans,* Mucoralean fungi, in remediation of xenobiotic, as well the microbial tolerance and resistance to dibenzothiophene, the present study was carried out to evaluate the physiological, morphological and ultrastructural aspects of the metabolism of *C. elegans* during the growth in presence of DBT.

2. Materials and Methods

2.1. Microorganism - *Cunninghamella elegans (UCP 596)* was obtained from Culture Collection of the University Catholic de Pernambuco, Recife, Brazil. The culture was maintained on potato dextrose agar tubes.
2.2. Cultural Conditions - The strain of *C. elegans (UCP 596)* was growth in Sabouraud dextrose agar medium (SAB) in Petri dishes, during 5 days at 28C. After this time, 10^7 spores/mL were collected and transferred for Erlenmeyer flasks of 500mL containing 100mL of Sabouraud dextrose broth medium, pH 6,0, incubated at 28ºC, in a reciprocating shaker for 96 hours, at 150Hz. The Solution Stock of Dibenzothiophene was prepared in dimethylformamide (DMF) concentration of 1M. Concentrations of DBT used in this paper were 0.50mM, 1.0mM and 2.0mM. All samples were prepared in three replicas.
2.3. Growth Curves - Samples collected at 24, 48, 72 and 96 hours, growth in the medium of culture Sabouraud Broth in the concentrations of DBT of 0.50mM, 1.0mM and 2.0mM were submitted to liophylization and maintained in a vacuum dissector until constant weight. The final value corresponded to arithmetic media of three replicas of each sample.
2.4. Morphological Studies - *C. elegans* was analyzed by the technique of microcultive described by [13]. The medium used was Sabouraud Agar, without DBT and in the different concentrations of dibenzothiophene

0.50mM, 1.0mM e 2.0mM. The Petri dishes were incubated at 28ºC, for observation of the alterations in the pattern of hyphae branching. Samples were observed and photographed by using light microscopy with amplification at 400X and 1000X.

2.5. Determinations of Glucose and Total Protein - Consumption of glucose was measure through enzymatic colorimetric method using (Kit Biosystems) for glucose oxidizes. For the determination of the total protein level was used the Kit Labtest, absorbance at 545nm.

2.6. Actin Cytoskeleton [14] - Samples collected at 24, 48, 72 and 96 hours of growth were washed quickly with phosphate-buffered saline (PBS; GIBCO), pH 7.2, and fixed for 5 min at room temperature (RT) with 5% Para formaldehyde in PBS. They were then permeabilized with 0.5% Triton X-100 in cold phosphate-buffered saline for 5 min, followed by three washes in PBS buffer without Triton, and a final wash with PBS. Labeling for actin was accomplished by 10 min RT incubation with a 1:20 dilution in PBS of FITC-conjugated phalloidin (Molecular Probes), followed by two 1 0-min washes with excess PBS. Labeled cover slip cultures were then placed cells-up on a glass slide, overlaid first with a drop of glycerol. Samples were observed and photographed in Microscope Axiostar Plus, Fluorescence ZEISS, HBO50/AC and photographed.

2.7. Scanning Electron Microscopy [15] - Samples collected at 24, 48, 72 and 96 hours were washed and fixed in 2,5% glutaraldehyde in 0,1M sodium cacodilate buffer at pH 7,4, washed, and post fixed with 0,1% osmium tetroxide, washed with 0,1 sodium cacodylate buffer, dehydrated in ethanol series 50%, 70%, 90%, 100%, 15 min each. Samples were mounted on aluminum stubs and observed and photographed by using a JEOL scanning electron microscope, model 5.600 LV, operating at 20 KV.

3. Results

Effects of DBT on the growth of *C. elegans* determinates by biomass production, consumption of glucose, pH and total protein are shown in Figure 1. It was observed a logarithmic growth of the control culture throughout during the experimental time, with a biomass production of 750mg/L. Culture treated with 0.5mM of DBT, yield a biomass production corresponding to 1.003mg/L, this value corresponding to an increase of 33.8% related to control culture. Cultures treated with 1.0mM and 2.0mM exhibited an increase of 24.0% and 19.4% compared with the control sample, respectively. The total glucose consumption was observed after 24 hours of growth for all treatments, including control. The pH values obtained revealed a pattern behavior similar for all treatments. The protein content increased in the first 72 hours of growth. After that a decrease in total protein content was detected for all treatments.

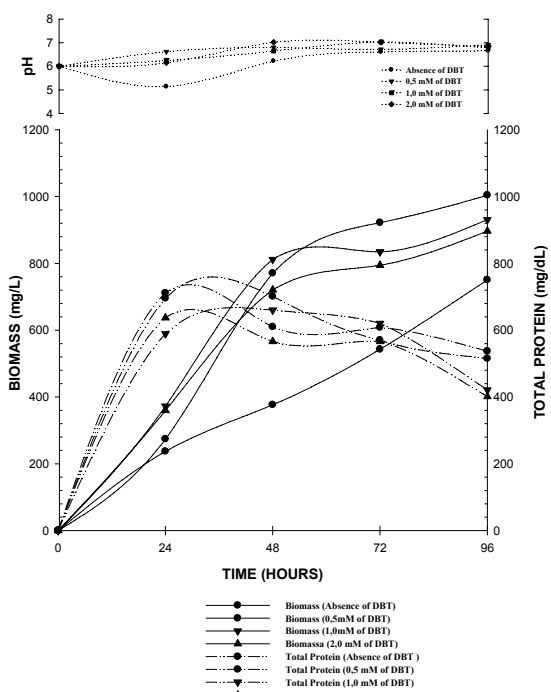

Figure 1. Growth profile of *C. elegans UCP-596* grown in medium Sabouraud control (without dibenzotiofeno) and medium Sabouraud treated with 0.5mM, 1.0mM and 2.0mM of dibenzothiophene, incubated at 28ºC, 150Hz, period of 96 hours determination of pH, determination of the biomass and total protein.

Results related to the morphology of *C. elegans (UCP-596)* samples, control and treated with DBT, are presented in the Figure 2A-D. Hyphae of control cultures exhibited a higher thickness, high transparency, and showed less branching, septum formation in the base of branching and absence of chlamidosporos (2A and 2A1). Culture samples treated with 0.5mM of DBT (2B and 2B1), showed tubular hyphae, thicker and dense, and homogeneous cytoplasm, an intense branching with different forms: wrinkled and bilateral. In culture samples treated with 1.0mM of DBT (2C and 2C1) are observed short branching development, with uniform texture and low density. In samples treated with 2.0mM of DBT (2D and 2D1) are observed an intense branching with short, slender, wrinkled and bilateral protrusions. The changes in the pattern of branching of hyphae of *C. elegans* are consequences of the dibenzothiophene presence in the culture medium. An intense and differential branching is observed and these changes are directly related to the DBT concentration, which result in a variation in the strategy for survival of the microorganism, with dispersion of the mycelium and uptake of nutrients in a limiting growth condition mediated by the presence of the xenobiotic.

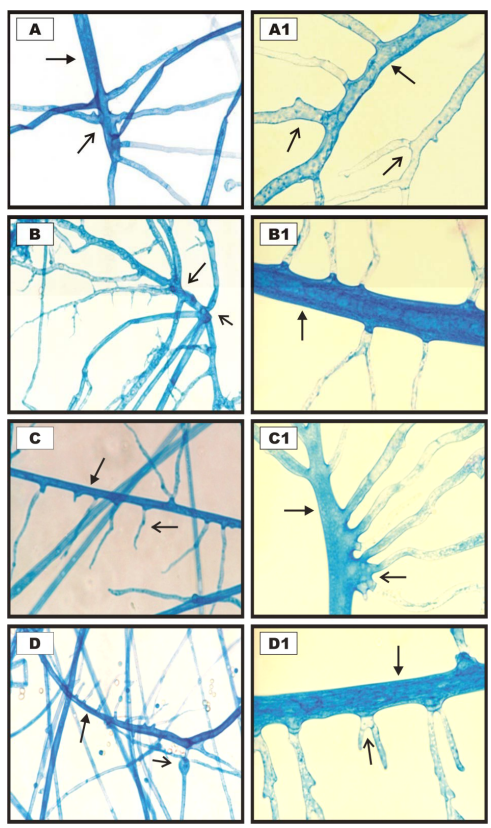

Figure 2. Variation of the patterns of branching of hyphae of *Cunninghamella elegans* UCP-596 where the figures (A, B, C and D increased of 400x) and (A1, B1, C1 and D1 increased of 1000x). Medium Sabouraud – Control (A and A1); medium of culture Sabouraud containing 0.5mM of DBT (B and B1); medium of culture Sabouraud containing 1.0mM of DBT (C and C1); medium of culture Sabouraud containing 2.0mM of DBT (D and D1). At 28ºC, 24 hours and 48 hours: hyphae (➜), branching of hyphae (➔).

The results obtained for the ultrastructural analysis *C. elegans* cells, control and treated with different DBT concentrations, collected at 24 and 96 hours are presented in Figures 3A-D. The results pointing that the presence of dibenzothiophene in the medium of culture influences the morphology of hyphae. Hyphae of *C. elegans* control cultures exhibited a tubular and elongated form, high electron density and few chlamidosporos are noted. Few branches are observed, figures 3A and 3A1, respectively. In samples grown in 0.5mM of DBT, collected in the intervals 24h and 96 hours, could be observed thin hyphae, homogeneous texture and low electron density, with a higher branching and higher chlamidosporos content than control samples can be observed in Figures 3 (B - B1). Samples de *C. elegans* mycelia grown in 1.0mM and 2.0mM of DBT are presented in Figures 3 (C and C1) and (D and D1), respectively. Irregular hyphae with low electron density, high transparency, with chlamidosporos varying size and shapes and intense branching are observed.

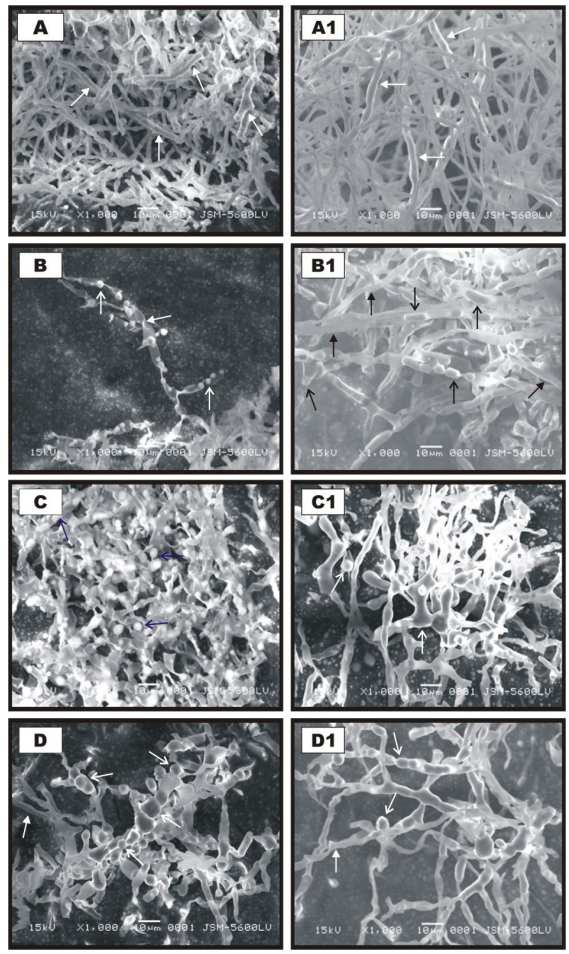

Figure 3. Eletronmicrograph to scan for observation of morphological changes of *Cunninghamella elegans* UCP-596, cultivated in the midst of culture Sabouraud Control (A and A1); medium of culture Sabouraud containing 0.5mM of DBT (B and B1); medium of culture Sabouraud containing 1.0mM of DBT (C and C1) and growing medium Sabouraud containing 2.0mM of DBT (D and D1), at 28º C, 48 hours. Body globosus (→); hyphae (➔).

The fluorescence study was conducted to visualize the possible effects of dibenzothiophene on the arrangement and distribution of the actin cytoskeleton in *C. elegans.* The results of fluorescence microscopy are shown in Figures 4A-D. In *C. elegans* hyphae grown without DBT, Figure 4 (A and A1), observed fluorescence outlining in the cell wall along of the hyphae in the regions of ramifications. Fluorescence staining in form of plaque and filaments in the cytoplasm, and transverse arrangements related to the longitudinal axis of hyphae are noted. Analysis of the micrographs of hyphae of *C. elegans* treated with 0.5mM of DBT determines differences compared with the control culture. An intense fluorescence is observed in the cytoplasmic region associated with the cell wall, which are essentially in the form of patches along of the hyphae. The sample shows a more intense staining in areas of branching and also in chlamidosporos compared the sample control. Mycelia of *C. elegans* treated with 1.0mM of DBT are showed in Figure 4 (C and C1). A low staining intensity along cell wall and in the form of small paths irregularly distributed in the cytoplasm was observed. Samples treated with 2.0mM of DBT, observed in Figures 4 (D and D1), exhibit an intense fluorescence outlining the cell wall uniformly, in branches and in the cytoplasm in patches form are observed.

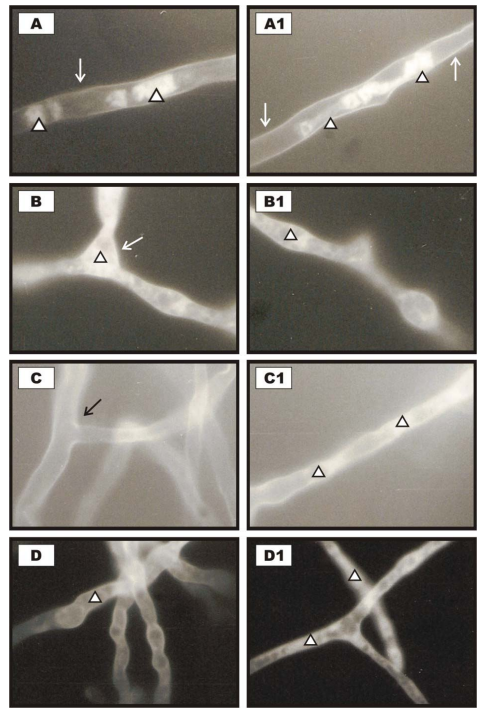

Figure 4. Microscopy Fluorescence: Samples of *Cunninghamella elegans* (UCP-596), control (A and A1); grown in 0.5mM of DBT (B and B1); grown in 1.0mM of DBT (C and C1); grown in 2.0mM of DBT (D and D1). Note the different forms of presentation of the actin cytoskeleton, with increase of 1000X: filaments and plaques in different areas of hyphae (△), cellular wall (→).

4. Discussion

Cunninghamella elegans has been reported to desulfurization DBT, which is oxidized first to DBT-5-oxide, then to DBT-sulfone, but is unable to further metabolize the sulfone to hydroxybiphenil derivatives. However, the oxidation of DBT to more water-soluble metabolites by *C. elegans* suggests that this fungus may play some role in the complete degradation of DBT, possible as part of a consortium of free-living organisms [12]. Studies related to the biochemical, physiological and structural behavior of microbial cells in presence of xenobiotic are essential in order to elucidate cellular mechanisms related to the resistance and tolerance for bioremediation purposes. Filamentous fungi are models for the study of innumerous cellular mechanisms and processes. Among them, those related to growth and morphogenesis is essentials. In mycelial fungi, hyphae extend by a highly polarized process of cell extension known as tip growth. It is reasonable pointed out that modifications in actin cytoskeleton could result in growth rate modifications and in alterations in cell morphology. Many xenobiotic can affect the microbial pattern growth. A search in the literature do not revealed studies on the effects of DBT on fungal growth or morphology. In this study the cellular aspects of growth and structure of *C. elegans* were analyzed in order to determine the effects of DBT. The results demonstrated the influence of DBT in the radial branching of mycelia, which could be related to the actin cytoskeleton modifications. Scanning electron microscopy and optical microscopy of *C. elegans* grown in the presence of DBT revealed structural changes in cells treated with the organo sulfur compound, leading to mycelia highly branched and shortened, and in a biomass production. These results are the first related to the DBT effects related to cell morphology.

5. Conclusions

C. elegans showed capable of growth in Dibenzothiophene in the concentrations 0.5mM, 1.0mM and 2.0mM. This study revealed the DBT effects on the cellular growth, and branching pattern of the organism in different concentrations, as observed by biomass production and by optical microscopy. The effects could be related to the actin cytoskeleton modifications observed by fluorescence microscopy. Also, the ultrastructural analysis demonstrated the hyphae modifications related to the texture, shape and electron density, presence of chlamidosporos, change in shape and size of the chlamidosporos by using scanning electron microscopy.

Acknowledgements: This work was carried out with the financial support of CAPES, FINEP / CTPETRO, CNPq and University Catholic de Pernambuco, Brazil.

Acknowledgements: the authors are grateful to CNPq, CAPES, and FINEP for financial support and fellowship.

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Evaluation of biosorption of Lead by *Halomonas eurihalina* strain NA-2

N. S. Ghazanfari[1], M. A. Amoozegar [2,*]

[1] Microbiology Lab., Dept. Of Microbiology, School of Biology, University College of Science, Science and Research Campus of Azad University, 1477893855 Tehran, Iran
[2] Extremophiles Lab., Dept. Of Microbiology, Faculty of Biology, College of Science, University of Tehran, 41556455 Tehran, Iran

[*]Corresponding author: e-mail: amozegar@khayam.ut.ac.ir, Phone: +98-21-61112622

Identification of toxic metal resistant strains is the first step in applying them in bioremediation. Among 24 halophilic strains isolated from salty environments in Iran, three showed better tolerance after MIC tests and as follow: NA-2, NA-5 and NA-7, were able to grow up to 5 mM of Pb2+ in sea water medium(SW-10). Among them, NA-2 was selected for further studies on bioremoval of Lead. According to Phenotypic characterization and 16 S r RNA sequence comparisons, this strain was more than 98% alike with Halomonas eurihalina.To study Lead bioremoval rate, after 48 hours of aerobic incubation in sea water medium containing 57 mg/L of Lead as Lead nitrate, Atomic Spectroscopy was used and results showed NA-2 strain dropped Lead concentration to 5.29 mg/L (90.71%). Biosorption studies were carried out in different temperatures, NaCl concentrations and pH values to study impact of these factors on bioremoval rate. Living biomass showed the best removal results in pH 5, 35°C and 5% NaCl while the exopolysaccharide showed best results in pH 5, 35°C and 10% NaCl.

Keywords Moderate Halophiles, Bioremediation, Lead tolerance, *Halomonas eurihalina*

1. Introduction

Metal pollutants can be produced through industrial processes such as mining, refining, electroplating, plastic manufacturing, fertilizers, pigments, metallurgical and petrochemical processes. [1], [2] at low concentrations, metals can serve as important components in life processes such as enzyme productivity. However, above certain threshold concentrations, metals can become toxic to many species. [1] Heavy metal pollution represents an important problem due to its toxic effect and accumulation throughout the food chain which leads to serious ecological and health hazards as a result of their solubility and mobility. Metal remediation through common physic chemical techniques is expensive and not eco-friendly. Hence, biotechnological approaches have received a great deal of attention as an alternative tool in the recent years. [3], [4] Unlike physical and chemical treatments, biosorption generally does not entail high operational costs and many potential sources of suitable biological material are cheaply and readily available. [5]

Since saline wastewaters are generated during the manufacture of chemicals such as pesticides, pharmaceuticals, and herbicides and during oil and gas recovery processes and conventional microbiological treatment processes do not function at high salt concentrations, therefore the use of moderately halophilic bacteria which have simple requirements for living and can tolerate unsuitable conditions should be considered. [3], [6] Halophilic microorganisms naturally need high ionic concentrations but non halophilic microorganisms do not have this ability and also increasing salt concentrations prohibits their growth while halophiles do not have this problem. [7]

Purpose of this study was to isolate resistant halophilic bacteria to lead and to study the possibility of applying strains with high MIC in bioremediation processes.

2. Materials and methods

2.1 Chemicals

Lead nitrate was obtained from Merck (E. Merck, Darmstadt, Germany). The stock solutions were prepared in distilled water and filter-sterilized by microbial filter (0.22 μm). Working solutions were stored at 45°C for approximately 5 days.

2.2 Isolation and cultivation

24 strains of moderately halophilic bacteria were isolated from salty environments including Gheshm, Garmsar, Isfahan, Karaj, Qom, and Tehran in Iran. All strains were grown in a saline nutrient broth (Merck) with a final total salt concentration of 100 gl^{-1} [8]. The salt solution composition was prepared as follows gl^{-1}:
NaCl, 81 ;$MgCl_2$, 7; $MgSO_4$,7 H_2O, 9.6; $CaCl_2$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.026.

When necessary, the medium was solidified by adding 15 g Agar (Merck). The cultures were incubated at 34 °C on an orbital shaking incubator (orbital incubator SI 50, Stuart Scientific) at 150 rpm for 48 hours. Among the strains isolated, three showed better tolerance to this metal ion. Strains NA-2, NA-5 and NA-7 could tolerate lead nitrate up to 5 mM; they grew well exhibiting a great resistance. The resistance of the strains to lead was determined by growing the bacteria in sea water medium containing 10% (w/v) NaCl at 34°C with the pH adjusted to 7.2-7.4 before autoclaving.

2.3 Identification of the isolate

Morphological and physiological characterizations were defined in basal culture media containing 10% NaCl (w/v). Gram reaction, motility, shape, and colour of colony, catalase, urease, oxidase activities, nitrate reduction, esculin, tween 20 and 80 hydrolyzes and indol production, acid production from carbohydrates and sugars and utilization of carbon and nitrogen sources was checked. [9] To determine the optimum temperature and pH for the growth of the strains, cultures were incubated at a temperature range of 5-55°C with intervals of 5°C and pH values of 5-11. Other physiological and biochemical tests were performed as described previously. [10] Genomic DNA of the isolate was extracted with a DNA extraction kit (Sigma) by following the manufacturer's recommended procedure. The 16 S r DNA gene of isolate was amplified using the universal primers 8F (5`-AGAGTTTGATCCTGGCTCAG) and 1541R (5`-AAGGAGGTGATCCAGCCGCA-3`). The amplification was done by initial denaturation at 95°C for 5 min followed by 10 cycles of 93°C for 1 min, 63°C for 1 min, 71°C for 1.5 min, 20 cycles of 93°C for 1 min, 67°C for 1 min, 71°C for 2 min and final extension at 71°C for 5 min. The purified PCR product was sequenced in both directions using an automated sequencer by SeqLab laboratory (Germany). The phylogenetic relationship of the isolate was determined by comparing the sequencing data with sequences of related *Halomonas* (Gene Bank database of the National centre for biotechnology information) and DNA-DNA hybridization was also performed. [11]

2.4 Determination of lead tolerance

The Agar dilution method was used to determine the tolerance of strains to lead [12]. Volumes of 20 ml of sea water medium containing 10% NaCl plus various concentrations of lead (0.3, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 and 30 mM) were poured into 8 cm plates. Then 10µl of the bacterial suspension (1.5×108 c.f.u ml^{-1}) was inoculated on each plate using a sampler followed by incubation at 34°C for 7 days. Minimum inhibitory concentration (MIC) for lead was determined.
Each plate was prepared in triplicates.

2.5 Lead removal experiments

Cells were cultured in 100 ml Erlenmeyer flasks containing 20 ml of sea water medium supplemented with 5% (w/v) NaCl (Basal medium) and 0.5 mM Lead nitrate. The basal medium was inoculated with 1% of the 1.5×10^8 c.f.u ml-1 of the bacterial suspensions and incubated aerobically at 35°C on a rotary shaker (150 rpm) for 2 days. The cells were pelleted out by centrifuging at 5000 rpm for 15 min and the supernatants were used for determination of the residual Lead, through atomic spectroscopy. According to results of MIC tests and also removal experiments, one strain was selected for further studies.

2.6 Evaluation of Lead removal by biomass, dead biomass and exopolysaccharide

Bacterial cultures were centrifuged at 5000 rpm for 15 min and cell pellets were washed with distilled water and centrifuged again and were ready to be used. In order to evaluate the removal rate by dead biomass, Erlenmeyer flasks containing bacterial cultures were autoclaved and then centrifuged at 5000 rpm for 15 min. Cell pellets were washed with distilled water and centrifuged again and were ready to be used. To study the removal by exopolysaccharide, after 3 days of aerobic incubation, bacterial suspension was centrifuged at 26000 rpm for 80 min at 4°C. The exopolysaccharide was then recovered by alcohol (Ethanol 96%) precipitation. One g of biomass, dead biomass and exopolysaccharide were brought into contact with 50 ml of pure Lead solutions. After 1 hour of exposure at room temperature on a rotary shaker, contents were centrifuged at 5000 rpm for 15 min and the supernatants were used for determination of the residual lead through atomic spectroscopy.

2.7 Factors effecting lead removal

To study the effect of different factors on removal rate, 1 g of living biomass and exopolysaccharide of the selected strain was added to 50 ml of pure Lead solutions. Exposure was performed at different pH values (3, 4, 5 and 6), temperatures (25, 35, 45 and 55°C) and NaCl concentrations (1%, 5% and 10%) on a rotary shaker (150 rpm) for 1 hour. Contents were then centrifuged at 5000 rpm for 15 min and the supernatants were used for determination of the residual Lead through atomic spectroscopy.

3. Results

3.1 Screening of moderately halophilic bacteria resistant to lead

Among many strains isolated from different salty environments in Iran, 24 resistant moderately halophilic bacteria were selected for further study. The intrinsic resistance of strains to lead nitrate was measured in the medium containing 10% NaCl and strains NA-2, NA-5 and NA-7showed higher MIC to Lead. They could grow up 5 mM of Pb^{2+}. Effect of different NaCl concentrations on resistance to lead was determined (Fig. 1).

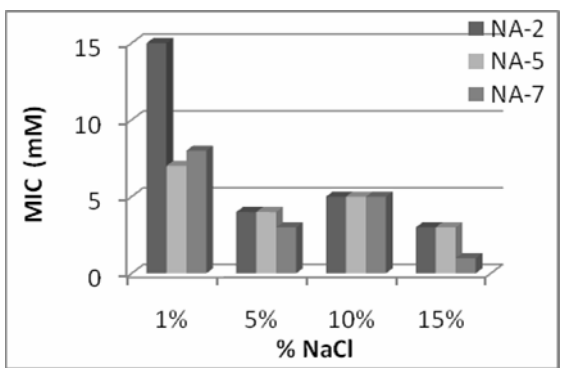


Fig. 1 Effect of salinity on resistance to lead in halophilic strains NA-2, NA-5 and NA-7 in sea water medium containing different NaCl concentration

After metal removal experiments in 3 resistant strains, NA-2 was shown to be a Gram-negative, non-sporulating, motile, facultative aerobic rod and produced catalase and oxidase. The colonies appeared round, smooth, mat and formed a creamy pigment and 2 mm size after 48 hours.

3.2 Determination of Lead removal in 3 strains with high MIC

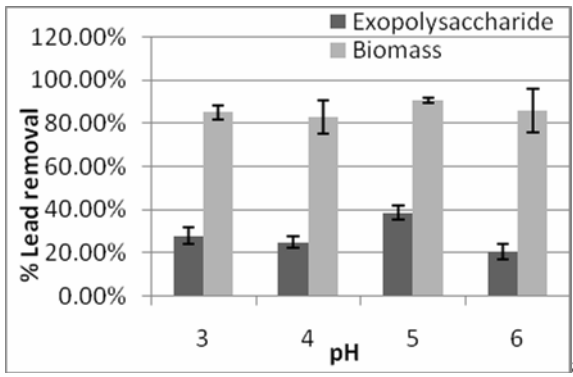
Lead removal by strains NA-2, NA-5 and NA-7 was studied. Maximum Lead removal in sea water medium containing 5% NaCl and 0.5 mM Lead nitrate was determined to be 90.71% by NA-2 strain after 2 days. According to MIC results and Lead removal experiments, NA-2 strain was selected for further studies.

3.3 Determination of Lead removal by living biomass, autoclaved biomass and exopolysaccharide of the selected strain

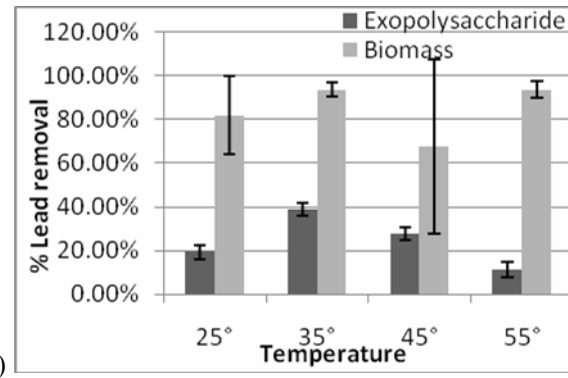
Lead removal by living biomass, autoclaved biomass and exopolysaccharide of NA-2 strain was measured and results showed maximum removal in case of using living biomass which was 91.27% after 1 hour of exposure then exopolysaccharide which was 41.19% and finally by autoclaved biomass which was 21.73%.

3.4 Effect of pH, temperature and NaCl concentration on removal rate

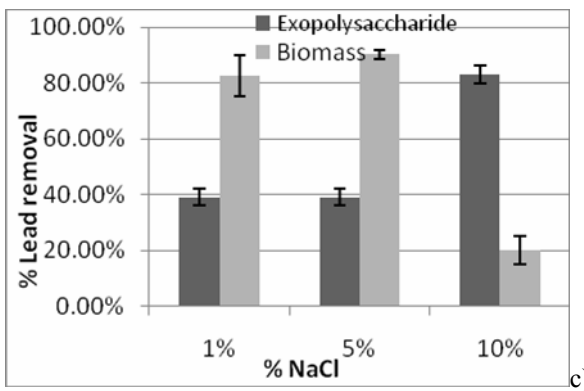
As shown in Fig. 2 a, effects of different pH value on lead removal were measured and maximum removal was occurred at pH 5 for both living biomass and exopolysaccharide. Effects of different temperatures on Lead removal were also measured and maximum removal occurred at 35°C for both living biomass and exopolysaccharide. (Fig. 2 b) Effect of different NaCl concentrations on the Lead removal was determined. Maximum removal was observed in the presence of 5% and 10% NaCl, for living biomass and exopolysaccharide respectively. (Fig. 2 c)



a)



b)



c)

Fig. 2 a) Effect of pH on lead removal by living biomass and exopolysaccharide of NA-2 strain. b) Effect of temperature on lead removal by living biomass and exopolysaccharide of NA-2 strain. c) Effect of different NaCl concentrations on lead removal by living biomass and exopolysaccharide of NA-2 strain.

4. Discussion

Moderately halophilic bacteria comprise a diverse group that grow in low-salt media and can tolerate high salt concentrations. [7] Identification of metal resistant strains is the first step in applying them in bioremediation processes [13]. Moderate halophiles are good candidates because they naturally need high anion and cation concentrations for their growth while other microorganisms don't have this ability and also increasing salt concentrations inhibits their growth and we don't see this problem in halophilic bacteria [7].

Among many moderately halophilic bacteria isolated from salty environments in Iran strain NA-2 showed the maximum MIC (equal to 5 mM), which was much higher than that previously reported for other moderate halophiles such as *Marinococcus halophilus* (2.5 mM) [7]. Effects of different NaCl concentration in the growth medium, on tolerance to lead were also evaluated. Decreasing NaCl concentration to 1% in growth medium led to higher tolerance of NA-2 strain to lead (15 mM).

Many researchers studied metal removal by bacteria and results showed their ability in removal of metals in culture medium. [3, 14, 2] Results reported for an unknown halophilic bacterium showed 70.48% and 44.98% removal capacity for Lead and Cadmium respectively after 2 weeks [15], which was much less than removal rate by D strain after 2 days.

Heavy metal ions can be entrapped in the cellular structures and subsequently biosorbed onto the binding sites present in the cellular structure. This method of uptake is independent of the biological metabolic cycle and is known as biosorption or passive uptake. The heavy metal can also pass into the cell a cross the cell membrane through the cell metabolic cycle which is the active way of uptake. Researchers have studied passive metal removal by dead biomass and active removal by living biomass and. For *Saccharomyces cerevisiae,* removal rate was 10 times greater when using living biomass [2]. Same results were obtained in our experience removal rate was much higher when using living biomass. Decreased removal rate by autoclaved biomass can be caused for two reasons: 1) in this case heavy metal attaches onto the surface of the cell wall and membrane and it has no penetration into the cell. 2) Some binding sites of the cell surface for the metal ions may be damaged or transformed as the result of autoclaving and its high temperature [16].

Experiments showed that in room temperature and by adding NaOH to adjust the pH of lead solutions, white precipitations of Lead hydroxide began to appear. To avoid this problem and subsequent faults we could only study effect of pH under value of 6 on the removal rate. Generally metal ion adsorption is pH dependent as the pH affects the availability of metal ions in solution, as well as the metal binding site onto cell surface [2].The temperature of the adsorption medium could be important for energy dependent mechanisms in metal biosorption. Energy-independent mechanisms are less likely to be affected by temperature since the process responsible for biosorption in this case seems to be largely physico chemical (electrostatic forces) in nature [2].

Same as in our experience where in removal by exopolysaccharide, temperature had no significant influence while in metal removal by living biomass, best results were obtained at 35°C. Best results were obtained at 5% NaCl which is close to optimum NaCl concentration for growth of NA-2 strain. But in the case of metal removal by exopolysaccharide, increasing NaCl concentration up to 10% led to best removal rate.

The increase of industrial activities has intensified environmental pollution problems. These polluted environments are often contained of different salts. Although bioremediation is usually one of the best processes for removing heavy metal pollutants, in salty conditions it seems to be difficult to proceed and active sludge activity reduces very much. Thus isolation of halophilic microbial strains such as NA-2 strain which is capable of resisting many toxic metal ions may be promising for bioremediation of contaminated saline soils and wastes discharge sites.

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Lactic acid bacteria: a potential tool to reduce ochratoxin A in wine

E. M. Mateo[1], A. Medina[1], F. Mateo[3], F.M. Valle Algarra[2], R. Mateo[2], and **M. Jiménez**[*,1]

[1] Dpto. de Microbiologia i Ecologia. Universitat de València. Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain.
[2] Dpto. de Química Analítica. Universitat de València. Dr. Moliner 50, E-46100, Burjassot, Valencia, Spain.
[3] Dpto. de Ingeniería Electrónica, Universitat Politècnica de València, Camino de Vera, 14. E-46022, Valencia, Spain.

[*]Corresponding author: Misericordia Jiménez Escamilla, e-mail: misericordia.jimenez@uv.es, Phone: +34 963543144

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by some species of the genera *Aspergillus* and *Penicillium*. OTA affects agricultural products all over the world and causes harmful effects on human and animal health because of its highly toxic properties. OTA has been detected in foods and beverages including grape juice and wine. New decontamination opportunities have been created by biological methods that involve elimination of mycotoxins by microorganisms. Several authors have observed that some bacteria and fungi show the ability to remove OTA. Reliable methods to reduce OTA level in food and beverages are highly desired to protect consumer health. The aim of this work was to assess the potential capability of some *Lactobacillus* species and *Oenococcus oeni*, all of them obtained from wine or must, to remove OTA from culture media. Two strains of both *Lactobacillus hilgardi,* and *L. paracasei sb. paracasei,* and 3 strains of both *L. brevis* and *O. oeni* were assayed. *Lactobacillus* spp. were grown in Man, Rogosa and Sharpe medium and *O. oeni* in Medium for *Leuconostoc oenos* (MLO). The culture media were spiked with 5 ng of OTA/ml. After incubation for 10 days at 28ºC, OTA level was determined by liquid chromatography. OTA removal in the cultures of *Lactobacillus* spp and *O. oeni* strains averaged 27.1% and 49.9%, respectively. Further studies were carried out with the most efficient *O. oeni* strains to evaluate the OTA reduction dynamics in MLO media spiked with 2 and 5 ng of OTA/ml. OTA was determined when cultures of the different strains arrived to 8 x 10^8 CFU/ml (time 0) as well as 5, 10 and 14 days later. OTA reduction levels ranged from 18.81% to 59.71% in the media contaminated with 5 ng/ml, and from 13.94% to 58.27% in the media contaminated with 2 ng/ml.

Keywords wine; mycotoxins; removal; food safety; mycotoxins; elimination; *Lactobacillus* spp; *Oenococcus oeni*

1. Background

Ochratoxin A (OTA) is well known for its toxic effects on biological systems and it has become more and more interesting since found to be widespread contaminant in various foods and beverages. OTA affects agricultural products all over the world and causes harmful effects on human and animal health because of its highly toxic properties. It has been reported as having mutagenic, teratogenic and carcinogenic activities [1].

OTA has been detected in foods and beverages [2─4] including grape juice and wine, where it was first reported by Zimmerli and Dick [5]. Since then, several surveys carried out in different countries have reported OTA levels in grape products and wine ranging from 0.01 to 3.5 μg/l. These levels were higher in products from southern regions of Europe than in northern regions [6─11].

According to the Codex Alimentarius, 15% of the total OTA intake is due to wine, which is considered as the second major source of OTA intake after cereals [12].

New opportunities involving the elimination of mycotoxins by microorganisms have been created and several authors have studied this subject and the ability to eliminate OTA has been observed for some bacteria such as *Acinetobacter calcoaceticus* [13], *Phenylobacterium immobile* [14], and some *Lactobacillus* [15]. *Saccharomyces cerevisiae* [16] and some *Aspergillus* species such as *Aspergillus niger*, *A. carbonarius* and *A. japonicus* [17, 18] are also capable of performing OTA degradation.

In grape must, up to ten lactic acid bacteria species can be identified. They belong mainly to the *Lactobacillus* and *Oenococcus* genera. Throughout alcoholic fermentation, a natural selection occurs and, finally, the dominant species is *O. oeni,* due to interactions between yeasts and bacteria and between bacteria themselves.

The aim of this work was to assess the potential capability of some *Lactobacillus* species and *Oenococcus oeni*, all of them obtained from wine or must, to remove OTA from culture media.

2. Material and Methods

2.1 Bacterial origin and growth conditions

Two strains of *Lactobacillus hilgardii*, 2 strains of *L. paracasei sb. paracasei,* 3 strains of *L. brevis* and 3 strains of *O. oeni* all of them isolated from Spanish wines were used. These strains were supplied by ENOLAB research group of the Department of Microbiology (University of Valencia).

2.2 OTA standard

A standard of ochratoxin A was purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain). It was diluted in acetonitrile-water-acetic acid (49.5:49.5:1, v/v/v) in sterile conditions to prepare a stock solution containing 100 µg of OTA/l. Appropriate volumes of this solution were added to the different culture media to reach the desired concentrations.

2.3 Evolution of OTA level in LAB cultures

The strains of *Lactobacillus* spp and *O. oeni* were grown at 28ºC without stirring until mid-log phase in Man, Rogosa and Sharpe medium (MRS) and Medium for *Leuconostoc oenos* (MLO), respectively.

The OTA stock solution was used to prepare MRS and MLO supplemented with 5 µg of OTA/l. As a preliminary study, 9-ml tubes containing MRS and MLO supplemented with 5 µg of OTA/l were inoculated with 50 µl of each *Lactobacillus* and *O. oeni* culture, respectively. Tubes were incubated at 28ºC and, after 10 days, OTA was determined by liquid chromatography (LC).

Subsequent studies were carried out in MLO with the strain of *O. oeni* that proved to be the most efficient in removing OTA from the medium to evaluate the reduction dynamics in media spiked with 2 and 5 ng of OTA /ml. The OTA stock solution was used to prepare MLO supplemented with 5 or 2 µg of OTA/l.

Nine millilitres of culture medium supplemented with OTA were poured in 10-ml transparent plastic screw cap sterile tubes, which were inoculated with 100 µl of each *O. oeni* culture and incubated at 28ºC. Aliquots were removed to quantify OTA by LC when the number of colony forming units per millilitre of culture medium (CFU/ml) was 8 x 10^8 (absorbance measured at 600 nm = 0.50), which was considered to be time 0, and 5, 10 and 14 days later. A control consisting of the same inoculum added to MLO without OTA addition was used and controls consisting in non-inoculated MLO spiked with OTA at the two levels were also run in parallel. All assays were carried out in triplicate.

2.4 Ochratoxin A determination

Tubes with bacterial cultures were shaken briefly and 0.6 ml sample of each tube was collected in sterile conditions and poured in Eppendorf centrifuge tubes, which were centrifuged at 4000 rpm during 6 minutes and 0.5 ml of supernatant was collected and used for OTA determination.

The proper amount of 0.1 M phosphoric acid solution was added to each sample so that the pH was in the range 2.5-3.0. Then, 2 ml of ethyl acetate were added. After shaking, the organic phase was loaded in an amber vial for LC analysis. This procedure was repeated once more time. After solvent evaporation under N_2 stream at 50 ºC, the residue was dissolved in 0.250 ml mobile phase. One hundred µl were injected into a LC-FLD system.

The LC system used consisted of a Waters 600E system controller, a Millipore Waters 717 Plus autosampler and a Waters 470 scanning fluorescence detector (Waters, Milford, Ma, USA). The excitation and emission wavelengths were 330 and 460 nm, respectively. The samples were separated using a C_{18} Phenomenex Gemini ® (150 x 4.6 mm, 5 µm) (Phenomenex, Macclesfield, UK), with a guard column of the same material. Run time for samples was 20 min with OTA being detected at about 12 min. The flow rate of the mobile phase (acetonitrile-water-acetic acid; 40:58:2, v/v/v) was 1 ml/min.

Calibration lines were constructed using culture media spiked with the proper amount of OTA standard in the range 0.2-5 ng of OTA/ml of medium. These analyses were made in triplicate.

Analysis of the results was carried out on a computer with Millennium® 4.0 software (Waters, Milford, Ma, USA).

2.5 Statistical analysis

The statistical analysis was performed using Statgraphics Centurion XV version 15.1.02 (StatPoint, Virginia, USA). Significant differences in mean values were reported at *p-value* < 0.05. Tukey-honestly significant difference (Tukey-HSD) multiple range test at 95% confidence level was used to group the cases into homogeneous groups with regard to the different parameters used.

3. Results and Discussion

3.1 Evolution of OTA level in LAB cultures

Fig. 1 shows the percentage of toxin reduction in the culture medium for the different strains of *Lactobacillus* spp and *O. oeni* in MRS and MLO, respectively, fortified with 5 µg/l after 10 days of incubation. Toxin level in the culture media was reduced in all the cultures but reduction was higher in cultures of *O. oeni*. After 10 days of incubation the *Lactobacillus* group and *O. oeni* showed average OTA reductions of 27.1% and 49.9%, respectively.

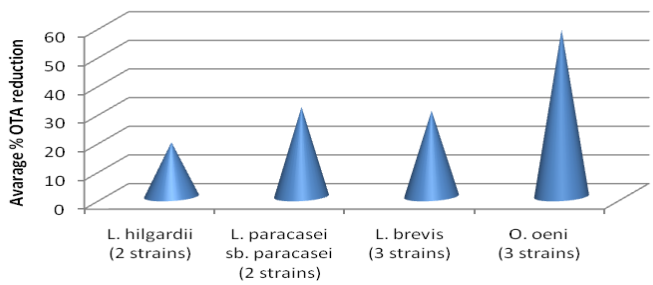

Fig. 1 Mean (%) of OTA reduction by the 4 lactic acid bacteria groups tested in culture media fortified with 5 µg of OTA/l after 10 days of incubation.

Fig. 2 shows the evolution of OTA level in MLO medium spiked with 2 and 5 µg OTA/l and inoculated with *O. oeni* (strain 6G) with time. OTA was determined at days 0, 5, 10 and 14 after inoculation. As can be seen, OTA reduction in cultures spiked with 5 µg of OTA/l ranged between 18.81% (day 0) and 59.71% (day 14). In MLO cultures fortified with 2 µg of OTA/l, the maximum reduction of OTA (58.27%) was observed at day 10. At the end of the incubation period (day 15) OTA reduction rate was 54%.

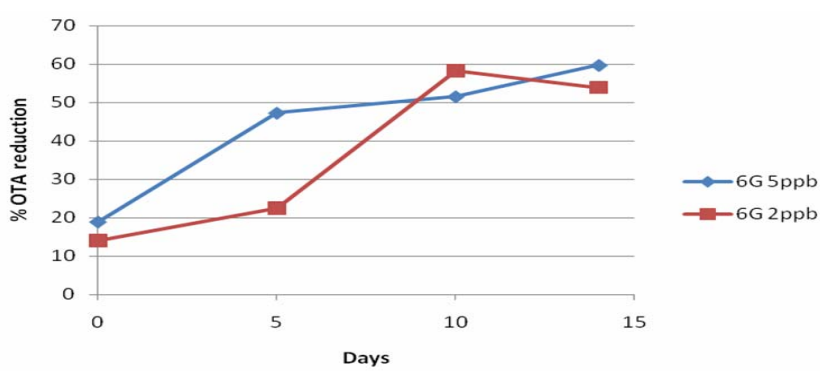

Fig. 2 Evolution of OTA level in MLO culture medium spiked with 2 and 5 µg of OTA/l, inoculated with *O. oeni* (strain 6G) isolated from wine and incubated at 28ºC.

In order to find out which factors significantly influence OTA reduction in the culture medium, data were studied by multifactor ANOVA. The factors used in this study were OTA level added to the culture medium (5 and 2 µg/l), and incubation time (time 0 and 5, 10 and 14 days after inoculation).

The statistical treatment of the data showed that only the factor time of incubation affected OTA removal percentage (*p*-value < 0.01).

Using Tukey-HSD multiple range test at 99% confidence level, the cases were grouped into homogeneous groups with regard to the different parameters used.

Incubation time split the cases into 3 homogenous non-overlapping groups. The first of the groups corresponded to time 0. The second group corresponded to the incubation period between time 0 and 5 days later. The third group corresponded to the 5-15 days period.

Several studies have reported the effect of some bacteria [19], fungi [20, 18] and yeasts [16, 21-23] on OTA reduction, but until now there has not been any study on the dynamics of OTA removal by *O. oeni*, the main species involved in malolactic fermentation (MLF) in wine.

Grazioli et al. [24] described OTA reduction during MLF in red wine winemaking using naturally contaminated grapes. In their study, OTA concentration was reduced during MLF in a range of 38% - 82.2% in two different type of wines. Our results suggest that *Lactobacillus* spp. and, specially, *O. oeni* are directly involved in this high OTA reduction and support the decontaminating effect of these lactic acid bacteria during winemaking.

4. Conclusion

The results of this study show that the 10 tested strains of lactic acid bacteria are capable of eliminating OTA from liquid media. *Oenococcus oeni,* the most important bacterium involved in the malolactic fermentation of wine, is the most efficient bacterium involved in the removal of OTA from the culture medium. OTA reduction rate is dependent on the incubation time (p-value < 0.01) but no significant differences were observed in OTA removal with regard to OTA concentration in the medium.

Acknowledgements The authors wish to thank financial support from the Spanish Government "Ministerio de Educación y Ciencia" (Projects AGL-2004-07549-C05-02/ALI and AGL2007-66416-C05-01/ALI and two research grants) and from the Valencian Government (Project GV04B-111).

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The Real-Time Behavior of Chromium in *Arthrobacter oxydans*

T. L. Kalabegishvili[1], A. N. Rcheulishvili, N. Y. Tsibakhashvili[*,1, 2], I. G. Murusidze[1]
S. M. Kerkenjia[2], O. A. Rcheulishvili[2], and H.-Y. N. Holman[3]

[1] Andronikashvili Institute of Physics, Tbilisi, Georgia
[2] Chavchavadze State University, Tbilisi, Georgia
[3] Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

[*]Corresponding author: e-mail: nelly_tsibakhashvili@yahoo.com, ntsiba@gol.ge, Phone: +995 32 396716, Fax: +995 32 391494

By combined application of electron spin resonance (ESR) and atomic absorption spectrometry the real-time behavior of chromium in *Arthrobacter oxydans* – a Gram-positive bacterium from contaminated Columbian basalt rocks (USA)- exposed to Cr(VI) was studied. The AAS spectrometry revealed the biphasic dynamics of chromium accumulation in bacteria under aerobic conditions. According to ESR measurements the time-course of Cr(III) hydroxide formation has the similar character. A kinetic model was proposed to describe this process. According to our results, *A. oxydans* accumulated part of chromium intracellularly.

Keywords *Arthrobacter oxydans*; ESR; AAS; chromium accumulation; Cr(III) hydroxide; bioremediation.

1 Introduction

Cr(VI) is one of the most common environmental metal contaminants released primarily by industries such as leather tanning, metal plating and alloying, and wood preservation. Cr(VI) compounds are toxic, highly water soluble species that move fairly rapidly in the subsurface [1]. The hazard of Cr(VI) can be mitigated by its reduction to less mobile and less toxic trivalent chromium Cr(III) compounds. Many bacterial strains isolated from contaminated sites hold the ability to reduce Cr(VI) to Cr(III) [2, 3]. These bacteria are under continual investigation and a deep molecular understanding has been gained for some of them. However, so far almost all studies have been focused on the behavior of Cr(VI) and a very few laboratory studies have concerned the direct determination of Cr(III) in the microbial systems. Until now, generation of Cr(III) complexes in the process of microbial reduction of Cr(VI) has not been investigated systematically. Moreover, little is known about the speciation of formed Cr(III) complexes, which is of considerable pragmatic importance because it dictates the long-term stability of reduced chromium in the environment, which is especially important in the development of new biotechnologies to remediate the sites contaminated with Cr(VI). This work is the continuation of our recent studies [4 - 6], where *Arthrobacter oxydans*, a widespread Gram-positive bacterium was used as our model. The purpose of the present work is (1) to study the nature and the real-time behavior of Cr(III) complexes generated in bacteria exposed to both low and high doses of Cr(VI); (2) to correlate these processes with chromium accumulation in bacteria.

2 Experimental

2.1 Bacterial growth conditions and sample preparation

A. oxydans was isolated from Columbia basalt rocks collected from 75 meters below the ground surface of the Eastern Snake River Plain in the United States of America [7]. These basalt rocks have been polluted with a mixture of heavy metals, radionuclides and organic compounds.
chromium concentration of 35 or 200 mg/L. Prior to the measurements, the bacterial cells were harvested at various time points by centrifugation (10 000 rpm, 15 min, 4^0 C) . The harvested bacterial cells were firstly washed in cold PBS (pH7) buffer. Then the PBS-washed pellet was resuspended in solution that contained 0.1 M Tris-HCl (pH 7.5) and 1.0 M EDTA and was incubated on a gyratory shaker at 100 RT for 2 h. From the Tris-HCl-EDTA medium, the bacterial cells were harvested by centrifugation (10 000 rpm, 15 min, 4^0 C) and were washed in PBS buffer**.** Electron spin resonance (ESR) spectroscopy was employed to monitor and to identify the formation of Cr(III) from Cr(VI). Atomic absorption spectrometry (AAS) was employed to study the dynamics of chromium accumulation in the bacterial cells. Prior to atomic absorption measurements, the samples were ashed with HNO_3.

All chemicals were ACS-reagent grade and purchased from Sigma (St. Louis, MO, USA).

2.2 Electron Spin Resonance (ESR) Experiments

The ESR measurements were carried out with the help of RE 1306 radiospectrometer (Russia) with computer-based digital systems for data acquisition and processing. The registration of Cr(III) was carried out at ambient (300 K) temperature as described previously [4]. Spectrometer settings: microwave frequency – 9.15 GHz, modulation frequency – 100 kHz, microwave power – 25 mW, time constant – 0.3 s. Field set – 3000 G, sweep width –2000 G, modulation amplitude – 20 G, scan time – 20 min.

2.3 Atomic Absorption Spectrometric (AAS) Experiments

The total chromium content in bacteria was measured using an atomic absorption spectrometer (Beckman 495, USA) with burner for acetyl-air flame. The detection was carried out at 357.9 nm.

3 Results and discussion

In our recent works [4-6], the nature and the formation of Cr(V) compounds during the reduction of Cr(VI) by *A. oxydans* was investigated systematically using ESR spectroscopy. The results of our batch experiments revealed that *A. oxydans* reduced Cr(VI) through the formation of Cr(V) complexes at the surface of bacteria in the presence of oxygen [4]. Numerical simulations of the ESR spectroscopic data provided strong evidence for at least two different diolato-type oxoCr(V) complexes ((**I,** g_{iso} = 1.9801; **II,** g_{iso} = 1.9796) involving bacterial cell wall macromolecules in the Cr(VI)–*A. oxydans* system [6]. According to our ESR measurements, Cr(V)-diols decomposed rapidly forming Cr(III) hydroxide (with a g –factor of 2.02 and a line width of 650 G). In this work we investigated the mechanism of Cr(VI) reduction to Cr(III) by *A. oxydans* with a focus on the time-dependent formation of Cr(III) complexes and uptake of chromium. The results are presented in Figs. 1 and 2.
Fig.1 depicts, that at the beginning of chromate action, the relative intensity of Cr(III) ESR signal, which is directly proportional to the concentration of Cr(III) hydroxide, increases rapidly and then remains almost unchangeable. Fig. 1 also illustrates that the Cr(III) formation dynamics proceeds differently at different doses of Cr(VI). Specifically, it saturates earlier at higher doses (200 mg/L).

To quantify the non-monotonous behavior of Cr(III) formation in bacteria, the following equation was successfully applied:

$$I = I_0 - A\exp(-k_f t) \qquad (1)$$

Here k_f is the first-order formation rate constant (days^{-1}), I_o and A are the fitting parameters.

The half-time of Cr(III) formation $(t_f)_{0.5}$ was calculated from $(t_f)_{0.5} = 0.693/k_f$. From our data, it was obtained (with the 95% confidence interval) that, for bacteria exposed to 35 mg/L and 200 mg/L of Cr(VI), $(t_f)_{0.5}$ = 3.5 and 3.7 days, respectively. The solid line in Fig.1 corresponds to the approximation carried out by the nonlinear Levenberg-Marquardt method.

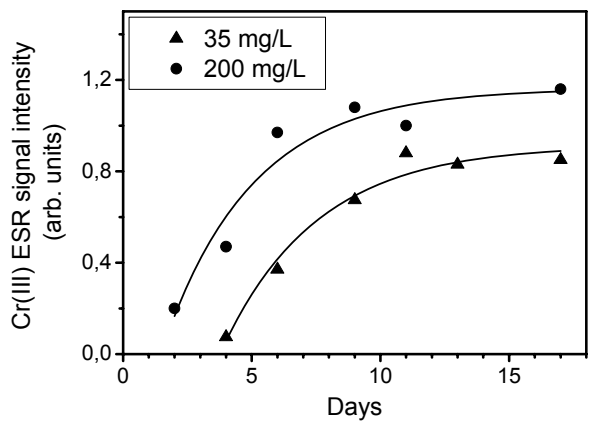

Fig.1. Time-course of Cr (III) formation in *A. oxydans.*
Bacterial cells were exposed to 35 or 200 mg/L of Cr(VI) action.

The time-course of Cr accumulation revealed the similar character (Fig.2). Besides, as it follows from Fig. 2, at both low and high doses of Cr(VI), the concentration growth in bacterial cells saturates after reaching the same concentration of about 10^4 μg/g. This value of Cr concentration was considered as critical for the *A oxydans* in our earlier work as well, where the dose-dependent formation of Cr(V) and Cr(III) was investigated [5].

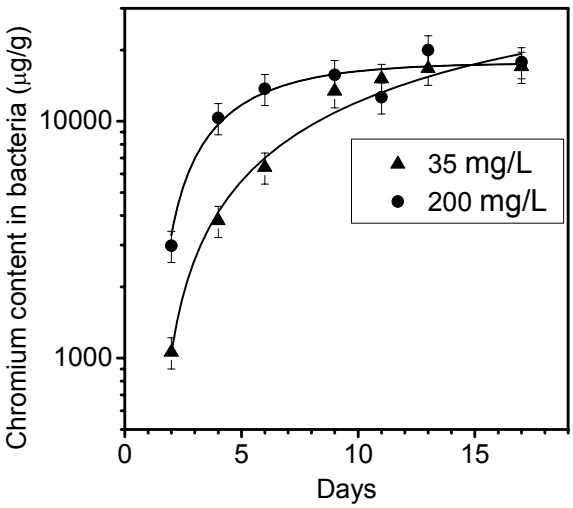

Fig.2. Time-course of chromium accumulation in *A. oxydans.*
Bacterial cells were exposed to 35 or 200 mg/L of Cr(VI) action.

The biphasic behavior of Cr accumulation indicates the presence of more than one uptake mechanism. To describe this process, we modeled the accumulation of chromium in the cell wall and in the intracellular space. In the model we assumed that Cr bound to the surface of bacteria was removed after intensive washing in ice cold Tris-HCl/EDTA buffer (experimental data are not presented here). Taking into account this circumstance, we described the chromium behavior in *A. oxydans* by the following set of equations [8] :

$$\frac{dW(t)}{dt} = k_{\mathrm{EW}}(E(c) - W(t)) - k_{\mathrm{WI}}W(t) \qquad (2)$$

$$\frac{dI(t)}{dt} = k_{\mathrm{WI}}W(t) - k_{\mathrm{II}}I \qquad (3)$$

Here E is the concentration of chromium in the external solution. W and I are the concentrations of total chromium in the cell wall and in the intracellular volume, respectively. k_{EW} and k_{WI} are the rate constants of the transfer of chromium from the external solution to the cell wall and from the cell wall into the intracellular volume.

Introducing the last term in the right-hand side of Eqn. (3), we modified the conventional model [8] valid for the processes when no saturation takes place. It followed from our AAS data, illustrating, that in the tested experimental system, some saturation was reached in chromium uptake by bacteria. The term $k_{II}I$ accounts for some intracellular process which decreases the rate of chromium accumulation with the rate constant k_{II} and, eventually, leads to the saturation of I. Indeed, the solution of the set of equations under the zero initial conditions, i.e., at the start of the experiment, $t = 0$, the concentrations $W = 0$ and $I = 0$, gives the following analytical expression for the total chromium (cell-wall + intracellular) content:

$$W + I = A\left\{1 + r_2 - \left(1 - r_1\right)e^{-t/d_1} - \left(r_2 + r_1\right)e^{-t/d_2}\right\} \qquad (4)$$

where

$$A = E\frac{k_{\mathrm{EW}}}{k_{\mathrm{EW}} + k_{\mathrm{WI}}} \qquad (5)$$

$$r_1 = \frac{k_{\mathrm{WI}}}{k_{\mathrm{EW}} + k_{\mathrm{WI}} - k_{\mathrm{II}}} \qquad (6)$$

$$r_2 = \frac{k_{WI}}{k_{II}} \quad (7)$$

$$d_1 = (k_{EW} + k_{WI})^{-1} \quad (8)$$

$$d_2 = k_{II}^{-1} \quad (9)$$

This function describes the process of saturation which is in $t >> \max(d_1, d_2)$. Our experimental data suggest that the saturation level of intracellular chromium is reached at the concentrations *I* which are lower than the concentration of total chromium in the cell wall *W*. Within the phenomenological model, Eqns. (2) and (3), this implies that $k_{II} > k_{WI}$. The solid lines in Fig.2 correspond to the curves modeling this process according to the solution of Eqn. (2) and (3). The characteristic times d_1 and d_2 are within the range of 1-4 days, which is in good agreement with the half-times of Cr(III) formation in *A. oxydans.* Detailed analysis of kinetic parameters (k_{EW}, k_{WI}, k_{II}) and also the appliciability of the proposed theoretical model for describing the chromium behavior in bacteria under different experimental conditions will be presented in our next work.

Thus, the time-course of Cr(III) hydroxide formation, as well as the Cr accumulation in *A. oxydans*, revealed the biphasic character, which could be described theoretically if we assume that part of chromium was accumulated inside the cells. This result is in accord with the experimental data obtained recently by Suzuki and Banfield [9]. They demonstrated that one of the high G+C Gram-positive isolates, closely related to *Arthrobacter ilicis,* accumulated uranium intracellularly as precipitates closely associated with polyphosphate granules. It is interpeted that sequestration of uranium into polyphosphate is a detoxification mechanism. As is known, heavy-metal resistant bacteria possess a number of strategies to withstand elevated concentrations of heavy metals [10]. The major bacterial resistance mechanisms include the exclusion by permeability barrier, efflux mechanisms, intracellular and extracellular sequestration, enzymatic transformations, the reduction of sensitivity of cellular targets to metal ions. Bacteria can use a few mechanims simultaneously to protect themselves. The establishment of the major survival mechanism of *A. oxydans* under our experimental conditions, as well as the main place of localization of Cr(III) inside the *A. oxydans* cells, needs future investigations. These experiments are underway.

Acknowledgements This work was funded by Grant # STCU-GNSFF07/131 (4330) from the Ukrainian Science and Technology Centre (STCU) and Georgian National Science Foundation (GNSF). The participation at the BioMicroWorld2007 was financed by GNSF Travel Grant # TR07/82.

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The role of Beta-Proteobacteria in aromatic hydrocarbon degradation: fingerprinting of 16S rRNA gene and catechol 2,3-dioxygenase gene by T-RFLP in BTEX degradative bacterial communities

András Táncsics[*,1], István Szabó[2], Sándor Szoboszlay[2], Erzsébet Baka[1], Károly Márialigeti[1], Sára Révész[1]

[1] Department of Microbiology, Eötvös Loránd University, H-1117 Budapest, Pázmány P. stny. 1/C, Hungary
[2] Department of Environmental Protection & Environmental Safety, Szent István University, H-2100 Gödöllő, Páter Károly u. 1., Hungary

[*]Corresponding author: e-mail: tancsics@elte.hu, Phone: +36 13812177

Detection of catechol 2,3-dioxygenase genes in aromatic hydrocarbon contaminated environments gives the opportunity to measure the diversity of bacteria involved in the degradation of these contaminants. In this study a primer set was designed to detect *Comamonadaceae* family (*β-Proteobacteria*) related catechol 2,3-dioxygenase genes in BTEX contaminated groundwater and diversity of these genes was investigated by terminal restriction fragment length polymorphism. Major differencies were observed in the microbial structure between the contaminated and the non-contaminated groundwater samples.

Keywords catechol 2,3-dioxygenase; BTEX degradation; T-RFLP; *β-Proteobacteria*

1. Introduction

Monoaromatic hydrocarbons, like benzene, toluene, ethyl-benzene and xylenes (BTEX compounds) are major contaminants of groundwater due to their frequent use in the industry. Bioremediation of the contaminated groundwater is a promising and cost-effective approach, therefore investigation of microbial communities involved in these processes is current today [1]. Many well known BTEX-degrading bacteria belong to the *Proteobacteria*, like *Pseudomonas* (*γ-Proteobacteria*) and *Sphingomonas* (*α-Proteobacteria*) species, which are widely investigated, and their substrate specificity and BTEX metabolizing pathways are well characterized. Whereas, there are also several aromatic hydrocarbon degrading bacteria in the group of *β-Proteobacteria*, like *Comamonas* and *Ralstonia* species. These latter species are believed to play a key role in the degradation of phenolic compounds rather than in BTEX degradation [2].

Bacterial communities involved in aerobic BTEX degradation are often investigated through the detection of key catabolic genes of this process, like catechol dioxygenases. The coding genes are often targeted by PCR primers and especially detection of catechol 2,3-dioxygenase (C23O) genes is in focus today [3]. These extradiol dioxygenase enzymes and their genes have a well-characterized phylogeny and can be divided into families and subfamilies [4]. The subfamily I.2.C is a quite heterogeneous group since a wide variety of bacterial genera can be found here, like *Comamonas*, *Ralstonia* and *Burkholderia* [1]. Due to the diversity of this group, C23O gene sequences show low similarity between each other, which does not allow designing subfamily specific primers for their detection. Therefore the diversity of C23O genes in subfamily I.2.C is less known.

A set of PCR primers were designed in this study to detect *Comamonadaceae*-related C23O genes. In this family well known aromatic hydrocarbon-degrading bacteria can be found and a lot of C23O gene sequence is known. These species are supposed to play a key role in phenol degradation via the *meta* cleavage [5] pathway but some of them are also able to utilize monoaromatic hydrocarbons [6]. The aim of this study was to detect these potential BTEX-degrading species and to reveal the diversity of them through the molecular fingerprinting characterization of C23O genes in a contaminated groundwater.

2. Materials and methods

2.1 Groundwater samples used in this study

BTEX contaminated groundwater samples were taken in 2007 in Hungary. Samples were collected into 1L sterilized bottles. Total Petroleum Hydrocarbon (TPH) concentrations of the samples were determined with the

WBSE-26:2006 standard method. Non-contaminated groundwater samples from the same sampling sites were used as controls. Dissolved oxygen concentration and redox potential were measured with WTW Handheld Meter Multi 350i (Nova Analytics Corporation, USA).

2.2 DNA extraction from samples

For the extraction of community DNA, 500 mL of the samples were filtered on sterile cellulose acetate membrane with 0.2 μm pore size (Millipore, USA). Filters were used for DNA extraction which was performed by MoBio UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., USA), according to the manufacturer's instructions.

2.3 C23O targeted PCR

To detect *Comamonadaceae* family related C23O genes, which belong to the I.2.C subfamily, new primers were designed. C23O gene sequences were obtained from GeneBank and the sequences were subjected to multiple alignment using the ClustalW algorithm and the MEGA3 software [7]. The following C23O gene sequences were retrieved from GenBank [8] (accession numbers are given in parentheses) to design primers: *Comamonas testosteroni* (AY568279), *Comamonas sp.* (U93090), *Acidovorax sp.* JS42 (CP000539), *Delftia acidovorans* (AB177545), *Delftia sp.* (DQ661649). The newly designed primers were COMC23O-F: 5'-CGA GAA CGT GCT GGG CAT GAA G-3', and COMC23O-R: 5'-AAG GCG ATG TCG TGC GGC-3'. The optimal estimated annealing temperature is 63 °C and the expected amplicon size is ~561 basepair long. PCR reactions were performed in a Perkin-Elmer model 2700 thermocycler according to the following temperature profile: 98°C (5 min), followed by 32 cycles of 94°C (0.5 min), 63 °C (0.5 min), and 72°C (1 min). The reaction was finished with an additional final extension for 10 min at 72°C.

2.4 Terminal Restriction Fragment Length Polymorphism (T-RFLP) investigations

For T-RFLP analyses, PCR reactions were performed using forward primers with a 5' fluorescent tag (HEX; Integrated DNA Technologies, Belgium). To obtain molecular fingerprints after amplification, subfamily I.2.C related C23O amplicons were digested with the restriction enzyme *Hin6*I (Fermentas, Lithuania) overnight at 37 °C. The reaction mixture contained 2.5 μL restriction enzyme buffer (Fermentas, Lithuania), 3U of restriction enzyme (Fermentas, Lithuania), 10 μL of template and ultra pure water to a final volume of 20 μL. After the digestion, samples were ethanol precipitated and resuspended in sterile distilled water. Digested DNA of 0.5–1.5 μL was added to 12 μL formamide and supplemented with 0.6 μL of DNA fragment length internal standard (Genescan TAMRA 500, Applied Biosystems, USA). Samples were then electrophoresed on an ABI 310 Genetic Analyzer (Applied Biosystems, USA), generating a plot of the size and the relative intensity of each fluorescent fragment.

2.5 Construction of C23O clone libraries

The I.2.C subfamily related C23O PCR products were purified (Viogene DNA/RNA Extraction Kit, Viogene BioTek Corp., Taiwan) and cloned into JM109 High Efficiency Competent *Escherichia coli* cells using the p-GemT Easy vector (Promega, USA). Transformants were selected with standard blue-white screening on plates supplemented by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Promega, USA), IPTG (Isopropyl β-D-1-thiogalactopyranoside**)** and ampicillin (Sigma-Aldrich, USA). Plasmid DNA was extracted with heat shock (98 °C, 5 min) and the inserts were reamplified with the specific primers designed in this study. PCR reagents and conditions were the same as described above. Clone libraries were screened by T-RFLP using the restriction enzyme *Hin6* (Fermentas, Lithuania) (see details above).

2.6 Sequencing and phylogenetic analysis

Unique T-RFLP ribotypes of the clone libraries were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA). Cycle sequencing products were analyzed with a model 310 automated Genetic Analyzer (Applied Biosystems, USA). The sequences were aligned with MEGA3 software initially against known sequences of the GenBank database (BLAST tool, NCBI). The sequence data of the closest relatives of the obtained clones were added to our alignment and phylogenetic trees were constructed with neighbor-joining method [9].

The partial catechol 2,3-dioxygenase gene sequences reported in this study are available from GenBank under accession numbers EU268278 to EU268283.

3. Results

3.1 Physico-chemical parameters of groundwater samples

At the contaminated area three groundwater wells were sampled. Two of them, the OM7 and the OM8 were highly contaminated with BTEX compounds. The groundwater originated from the OM3 well was slightly contaminated and only low benzene contamination was found here while the concentrations of the other BTEX compounds were under the detection limit. This latter sample was used as a background or non-contaminated sample during the study. All of the wells aerobic conditions prevailed and dissolved oxygen concentrations were about 4 mg L^{-1}. Table 1 shows the contamination parameters of the investigated groundwater samples. The aerobic condition of the groundwater was ensured by a remediation process. Three months before the sampling, when the remediation process was started, hypoxic conditions were found in the contaminated wells (dissolved oxygen concentrations 0.2 mg L^{-1}) while the non-contaminated well aerobic condition prevailed (dissolved oxygen concentration 2.9 mg L^{-1}).

Table 1 Characteristics of investigated groundwater samples

Samples	Concentration in the groundwater (μg L^{-1})					Dissolved O_2 concentration (mg L^{-1})	Redox potential (mV)	Depth (m)
	Benzene	Toluene	Ethyl-benzene	Xylenes	TPH			
OM3	77,9	<1	<1	<1	<50	3,71	228	3,16
OM7	4141	2480	117	1420	2150	3,60	153	2,81
OM8	1990	1580	69	1570	3200	4,32	212	2,72

3.2 PCR detection of C23O genes in the samples

Subfamily I.2.C related C23O genes were recovered from every DNA extract. This means that species harboring these genes were present not only in the contaminated but in the non-contaminated sample as well. All PCR product lengths matched the expected fragment size. Non-specific fragments were not detected. Since preliminary studies had shown the lack of *Pseudomonas*- and *Sphingomonas*-related C23O genes, our results suggested that mainly subfamily I.2.C related C23O genes possessing bacteria were responsible for BTEX degradation in the investigated groundwater samples The fact, that these bacteria were present in the non-contaminated samples as well, enforced us to perform T-RFLP analysis with all of our PCR products. It was questionable, whether the same C23O genes are present in all of the samples, or there are significant differences between the contaminated and the non-contaminated samples.

3.3 T-RFLP investigations of C23O products

After digestion of HEX labeled subfamily I.2.C related C23O PCR products with the restriction enzyme *Hin6*I, samples were analyzed with capillary electrophoresis. Figure 1 shows the obtained chromatograms. Three different fragments were present in each sample but the fragments obtained with the non-contaminated sample significantly differ from those fragments which were obtained with the contaminated samples. This means that other types of C23O genes within the subfamily I.2.C were dominant in the contaminated groundwater than in the non-contaminated. This could be a result of differences of BTEX concentration or due to other physicochemical environmental variables, like change of dissolved oxygen concentration, pH or redox potential due to the contamination.

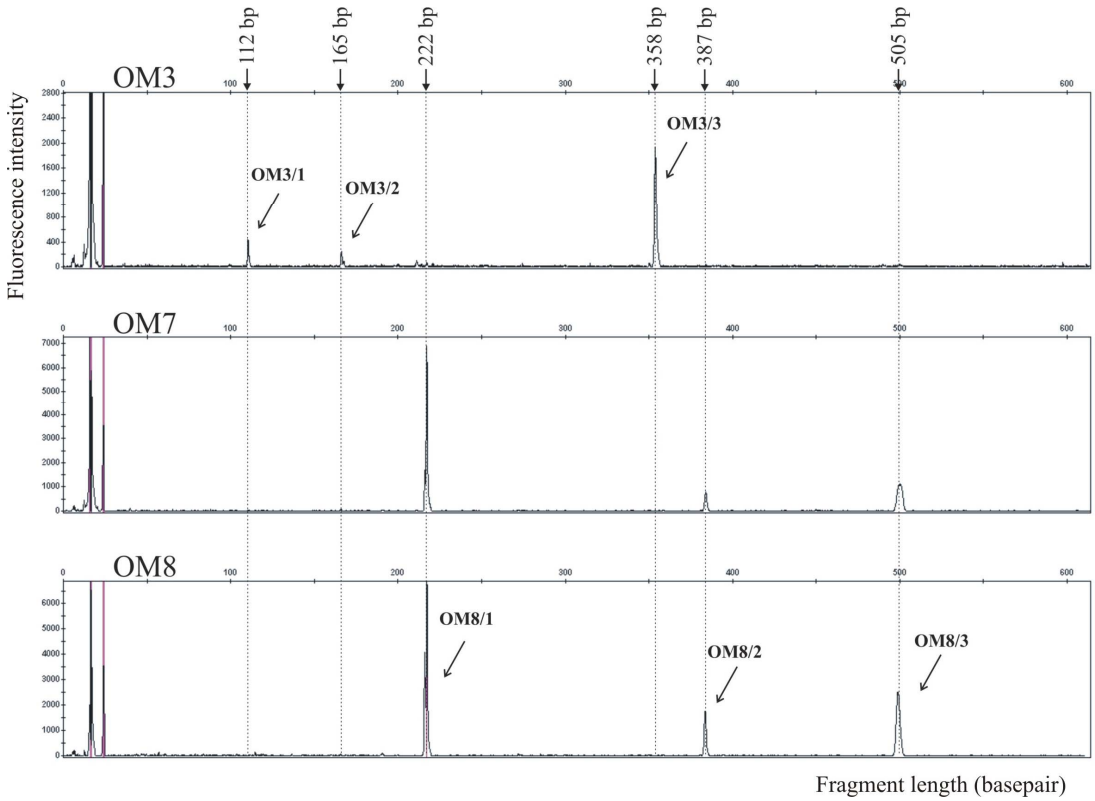

Fig. 1:T-RFLP profiles of detected C23O genes

3.4 Results of C23O clone libraries

To link taxonomic identity to the T-RFLP fragments, the C23O PCR products of OM3 and OM8 samples were cloned and sequenced. All of the fragments were identified and the obtained clone sequences were found to show low similarity to other subfamily I.2.C related C23O gene sequences. Similarity values varied between 75 – 86%. Phylogenetic analysis based upon the nucleotide sequences and deduced amino acid sequences showed, that all of the detected genes belonged to the I.2.C subfamily of extradiol dioxygenases.

4. Discussion

In this study a new primer pair was designed and tested to detect mainly *Comamonadaceae* family related C23O genes inside of the subfamily I.2.C of extradiol dioxygenases .Moreover, the distribution of different genotypes of these extradiol dioxygenase genes was investigated in contaminated and in non-contaminated aquifers by T-RFLP. It was found that subfamily I.2.C related C23O genes were dominant in all analyzed groundwater samples but different genotypes of these C23O genes were present in the contaminated samples and in the non-contaminated sample. The dominancy of I.2.C subfamily related C23O genes was unexpected and may have been caused by former hypoxic conditions of the contaminated groundwater. As Kukor and Olsen suggested in their study, C23O enzymes in subfamily I.2.C possess a greater affinity for molecular oxygen [10] and this phenomenon could cause the dominancy of these genes in the investigated groundwater. It is striking, that different genotypes of these genes can be found in the contaminated and in the non-contaminated samples. This radical change can be explained with the high BTEX contamination and with the low dissolved oxygen concentration of the groundwater originated from OM7 and OM8 wells. This change is obvious because those bacteria will be dominant in these contaminated environments which possess such C23O enzymes which were adapted to these conditions. This adaptation or evolution of C23O enzymes has been investigated recently in some studies. Junca *et al*. [11] found in their study that a single amino-acid substitution can cause the change of kinetic properties of C23O enzymes, even if, this substitution does not influence the active site structure directly. Directed evolution experiments of Suenaga *et al.* [12] showed that substrate specifities of aromatic hydrocarbon dioxigenases can be changed by a single amino-acid substitution.

In conclusion the subfamily I.2.C related C23O genes could be dominant in the investigated BTEX contaminated groundwater because of the grater affinity for molecular oxygen of the coded C23O enzymes and, due to this, *β-Proteobacteria* may have important role in BTEX degradation in hypoxic environments.

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Biosurfactants: Purification, Mass Production, Applications

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Applications of Surface Active Compounds by *Gordonia* in bioremediation and washing of hydrocarbon-contaminated soils

P. Caredda[1], **A. Franzetti**[*1], **M. Papacchini**[2], **P. La Colla**[3], **C. Ruggeri**[3], **E. Tamburini**[3] and **G. Bestetti**[1]

[1] Dept. Environmental Sciences, University of Milano – Bicocca, 20126 Milano, Italy
[2] ISPESL, Dept. for Production Premises and Interaction with Environment, via Fontana Candida 1, 00040 Monteporzio Catone (RM), Italy
[3] Dept. Biomedical Science and Technology, University of Cagliari, Cittadella Universitaria, Monserrato (CA), Italy.

[*] Corresponding author: Andrea Franzetti e-mail: andrea.franzetti@unimib.it, Phone: +39 2 64482927

Recently we isolated two *Gordonia* sp. strains able to produce two different types of SACs (surface-active compounds): extracellular bioemulsan(s), able to produce stable emulsions but not to reduce surface tension, and biosurfactant(s), able to reduce surface tension. The aim of this work was to evaluate the potentialities of the strains and their synthesised products in bioremediation and soil washing technologies. Microcosm bioremediation experiments were carried out with aliphatic hydrocarbon contaminated soil, while batch soil washing experiments were carried out with crude oil contaminated soil. Bioremediation results showed that the bioemulsan is able to reduce final concentration of recalcitrant branched hydrocarbons. On the other hand, results from soil-washing experiments demonstrated that the bioemulsan effectively removes crude oil from soil. Overall results are encouraging for a field scale application of SACs by *Gordonia* in soil remediation.

Keywords biosurfactant; bioemulsifier; bioremediation; soil-washing; hydrocarbon

1. Introduction

Due to their properties, microbial surface active compounds (SACs) have been exploited in environmental remediation techniques. Rhamnolipids and surfactin have been investigated for application both in biological and chemico-physical technologies for the remediation of soil and water contaminated by hydrocarbons and heavy metals.

Biosurfactants can enhance the biodegradation rate of hydrocarbons by two mechanisms, (i) enhanced solubility of the substrate for the microbial cells, and (ii) interaction with the cell surface, which increases the hydrophobicity of the surface for an easier access to hydrocarbons [1,2]. Zhang and Miller [1] showed that a concentration of 300 mg/L of rhamnolipids increased the mineralization of octadecane from 5% to 20%. The rhamnolipids increased the solubility of the hexadecane from 1.8 to 22.8 mg/L even if there have been indications that inhibition can also occur. Rahman et al. [3] demonstrated a beneficial effect of rhamnolipids for high-molecular weight hydrocarbons (C32-C40) in soil microcosms [4,5]. The interior of a micelle constitutes a compatible environment for hydrophobic organic molecules; the process of incorporation of these molecules into a micelle is known as solubilisation [6]. Surfactants have been applied for stimulating the dissolution of non-aqueous phase liquids initially present in soil [7,8], the dissolution of solid contaminants [9], and the desorption and transport of soil-sorbed contaminants [10].

Rhamnolipids have been used to remove oil from porous matrixes. They released three times as much oil as water alone from the beaches in Alaska after the Exxon Valdez tanker spill [11]. Van Dyke et al. [12] had, previously, found that they could remove, at a concentration of 5 g/l, approximately 10% more hydrocarbons from a sandy loam soil than a silt loam soil and that sodium dodecyl sulfate (SDS) was less effective than the biosurfactants in removing hydrocarbons.

We recently isolated two *Gordonia* sp. strains (BS29 and M22) that extensively grow on aliphatic hydrocarbons and are able to produce SACs. In particular, *Gordonia* sp. BS29 produces at least two different types of SACs: extracellular bioemulsan(s), able to produce stable emulsions but not to reduce surface tension, and biosurfactant(s), able to reduce surface tension [13]. The aim of this work was to evaluate the potentialities of the strains and of the synthesised products to enhance bioremediation of hydrocarbon contaminated soil.

2. Materials and Methods

2.1 Preparation of crude bioemulsan

For preparation of bioemulsan *Gordonia* sp. BS29 cultures were prepared in M1(per litre: K_2HPO_4 1.32 g, KH_2PO_4 1 g, NH_4Cl 0.81 g, $NaNO_3$ 0.84 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4$ 0.20 g, $CaCl_2$ 0.02 g) medium at initial OD_{600} of 0.05 and n-hexadecane (2% w/v) as carbon source. After incubation (200 rpm) at 30° C cultures were centrifuged (7000 rpm for 15 min) and the biomass was mechanically removed. The cultures were then filtered on sterile membrane (0.45 μm) to remove residual bacteria. The filtrate was subsequently lyophilized in order to obtain a solid residue (crude bioemulsan).

2.2 Bioremediation experiments

Two different experimental sets of soil microcosm have been designed. Uncontaminated garden soil was amended with inorganic nutrients (N, P) and contaminated with single aliphatic hydrocarbons in experimental set 1 and with single aromatic hydrocarbons in experimental set 2. Soil was contaminated dissolving the contaminants in n-hexane and adequately mixing the solvent and the soil (0.5 ml of n-hexane per gram of soil). After solvent evaporation, soil samples were analyzed for their hydrocarbon concentrations. Triplicate microcosms were prepared for each condition. Each microcosm consists in 50 g of contaminated soil in an aerated serum bottle. Bottles were kept al laboratory temperature and soil humidity was maintained constant by de-ionized water addition (25%). At fixed kinetic time soil samples were removed and the residual hydrocarbon concentration was determined.

Two g of soil samples were added with 30 ml n-hexane containing *o*-terphenyl as internal standard. The bottles were sealed with a Teflon stopper and held for 30 min in an ultrasonic bath at 47 kHz frequency; the extracts were filtered on anhydrous sodium sulphate, dried, dissolved in hexane, analysed and quantified by internal calibration.The analyses were performed with an Agilent 5890 gas chromatograph coupled to a FID detector with HP5 column (30 m length, 0.32 mm i.d., 0.25 μm film thickness). The temperature program was 2 min at 40°C, then 12°C/min to 300°C, and 5 min at 300°C. Injector and detector temperatures were set to 250°C.

2.3 Soil washing

Garden soil was artificially contaminated dissolving crude oil in n-hexane and adequately mixing the solvent and the soil to reach the concentration of crude oil in soil of 10% w:w. Soil samples were left uncovered for 48 hours to permit evaporation of the solvent and volatile components of the crude oil.
Batch soil washing experiments were carried out in sealed bottles containing 2 g of soil and the suitable washing solution, according to the experimental design. Bottles were shaken at 200 rpm for the suitable washing time.
After the washing, the bottles were centrifuged at 2000 rpm for 5 minutes, the supernatant solution was discarded, the soil was rinsed with water and centrifuged again. After discarding the supernatant solution, the soil was dried with anhydrous sodium sulphate and extracted four times with 10 ml of n-hexadecane. The extracted crude oil was evaluated by absorbance measurement (400 nm). The crude oil removal was determined by difference between the absorbances of washed and unwashed soil.

2.4 Data Analysis

ANOVA analyses of experimental design results and regression analyses have been performed using STATISTICA software.

3. Results

3.1 Bioremediation of aliphatic hydrocarbon contaminated soil

Triplicate soil microcosm experiments have been set up with the following conditions: (1) without any addition (TQ), (2) *Gordonia* BS29 addition (10^7 UFC/g) (BS), (3) *Gordonia* M22 addition (10^7 UFC/g) (M22), (4) addition of crude bioemulsan at high concentration (2000 mg/kg) (BEH), (5) addition of crude bioemulsan at low concentration (200 mg/kg) (BEL), (6) addition of rhamnolipids mixture (40 mg/kg) (R). Each microcosm consisted in 50 g of aliphatic hydrocarbon contaminated soil in a 125 ml-glass bottle. Soil was preliminary contaminated with the following compounds at a concentration of 300 mg/kg (each contaminant): n-hexadecane,

n-heptadecane, pristane, n-eicosane, n-octacosane, squalene. Over time, the analyses of residual hydrocarbons, total microorganisms and hydrocarbon-degrading microorganisms were carried out at the beginning of the experiments and after 8, 19 and 53 days.

Hydrocarbon biodegradation data were analysed by Analysis of Variance (ANOVA) to compare the tested condition compared to the condition without any addition. The dependent variables for the comparison were the biodegradation rate (k) and the residual concentration of each contaminant after 53 days of experiments. The biodegradation rate was computed as the first order kinetic constant of the exponential equation best fitting experimental data.

3.1.1 Data analysis

ANOVA tests were carried out to evaluate the significance of the differences of the mean between each treatment and the control TQ both for residual concentration and biodegradation rate. The F Test, used as diagnostic test for ANOVA, allowed us to evaluate whether the treatment led to a significant enhancement of the rate and/or the extent of biodegradation of the tested compounds. Since the significance of the test was fixed at 0.05 and the sums of squares were calculated on three values, thus the critical F value is $F_{0.05,1,4} = 7.70$. If calculated F values are greater than critical F values, the difference between the means can be consider significant.

Tables 1 and 2 show the means and the calculated F values of ANOVA test for residual concentration and biodegradation rate, respectively.

It is evident that both bioaugmentation treatments (BS and M22) did not lead neither to an increase of biodegradation rate nor to a reduction of residual concentration for any contaminants. In some cases the addition of exogenous bacteria led to a reduction of biodegradative capacity of the entire community. For most of the contaminants even the addition of rhamnolipids, did not result in the enhancement of biodegradation. However, ANOVA test showed that both the addition of rhamnolipids and bioemulsan reduced the final concentration of pristane in respect to the control. Pristane can be considered a model compounds for highly branched and recalcitrant compounds. It is known as a residual contaminant in several bioremediation application of diesel fuel contaminated soil. Furthermore, the bioemulsan increased also the biodegradation rate of pristane. Summarising the results from data analysis, we found that the extracellular bioemulsan seems to have positive effect on the rate of biodegradation and residual concentration on branched aliphatic hydrocarbons in contaminated soil.

Table 1. Results of ANOVA test for residual concentration (mg/kg) in biodegradation experiments with aliphatic hydrocarbons. Each treatment is compared with the control TQ. F value in bold indicates significant differences of the means (Fcrit $_{0.05,1,4}$=7.7).

	n-hexadecane TQ mean= <dl		**n-heptadecane TQ mean = <dl**		**pristane TQ mean = 4.3**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	<dl	nd	<dl	nd	5.6	4.19
M22	<dl	nd	<dl	nd	4.3	<0,05
BEH	<dl	nd	<dl	nd	2.8	**10,7**
BEL	<dl	nd	<dl	nd	6.0	**9,0**
R	<dl	nd	<dl	nd	1.3	**45,5**

	n-eicosane TQ mean = 3.8		**n-octacosane TQ mean = <dl**		**squalene TQ mean = <dl**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	0.4	3.92	<dl	nd	2.3	0.44
M22	5.6	6,9	<dl	nd	<dl	nd
BEH	4.6	8.3	<dl	nd	<dl	nd
BEL	5,1	0,5	<dl	nd	<dl	nd
R	2.07	0.5	1	nd	<dl	nd

Table 2. Results of ANOVA test for biodegradation rate (days-1) in biodegradation experiments with aliphatic hydrocarbons. Each treatment is compared with the control TQ. F value in bold indicates significant differences of the means (Fcrit $_{0.05.\ 1.4}$=7.7).

	n-hexadecane TQ mean= - 0.279		**n-heptadecane TQ mean = - 0.264**		**pristane TQ mean = - 0.137**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	-0.3	<0.05	-0.3	<0.05	-0.12	2.1
M22	-0.219	**362.0**	-0.208	**367.1**	-0.128	**11.1**
BEH	-0.24	1.9	-0.250	8.0	-0.146	**13.5**
BEL	-0.151	**1725.2**	-0.146	**1218.0**	-0.093	**131.4**
R	-0.26	0.8	-0.25	0.8	-0.12	1.3

	n-eicosane TQ mean = -0.256		**n-octacosane TQ mean = -0.20**		**squalene TQ mean = -0.206**	
	mean (mg/kg)	**F**	**mean (mg/kg)**	**F**	**mean (mg/kg)**	**F**
BS	**-0.19**	**47.2**	-0.18	1.9	-0.20	0.2
M22	**-0.223**	**54.8**	-0.200	0.1	-0.202	1.4
BEH	**-0.17**	**53.1**	-0.201	0.3	**-0.142**	**25.3**
BEL	**-0.153**	**464.1**	-0.189	1.0	**-0.155**	**355.4**
R	**-0.225**	**30.6**	-0.190	0.8	-0.193	3.8

3.2 Washing of crude oil contaminated soil

To evaluate the applicability of extracellular bioemulsan in soil washing treatment a set of experiments was designed. Soil was artificially contaminated with crude oil (10% w:w) and batch washing experiments were carried out in different conditions. A $3^{(3)}$ three level full factorial design (FFD) with six central points (33 experiments) was chosen with one qualitative variable and two quantitative ones. The qualitative variable was the washing solution while the two quantitative ones were the ratio between soil and washing solution, and the time of washing. Table 5 shows the variables and the levels chosen for this experimentation.

Table 3. Variables and values of the levels in $3^{(3)}$ FFD

VARIABLE/LEVEL	**LOW**	**MEDIUM**	**HIGH**
Washing solution	Water	Bioemulsan (2% w:v)	Rhamnolipid mixture (2% w:v)
Ratio washing solution:soil (v:w)	10	15	20
Time of washing (min)	30	50	70

Residual crude oil concentration after washing was measured by spectrophotometric analysis of n-hexane extract from soil and the percentage of crude oil removed was calculated for each conditions. A complete list of the crude oil removal in the experiments is reported in Annex 1.

3.2.1 Data analysis

ANOVA tests were used to evaluate the significance of the effect of the bioemulsan and rhamnolipids on crude oil removal compared to water. Furthermore ANOVA was used within the experiments with the same washing solution to individuate the factors between soil:washing solution ratio and time of washing that significantly affect the removal.

Table 4 shows the results of ANOVA test for the comparison of bioemulsan with only- water treatment.

Table 4. ANOVA results for bioemulsan washing of oil contaminated soil

Groups	*n*	*Sum*	*Mean*	*Variance*
Water	9	175.523	**19.5**	99.33818
Bioemulsan	9	387.343	**43.0**	53.97843

ANOVA

	SS	*df*	*MS*	*F*	*p*	*F crit*
Between groups	2492.6	1	2492.6	**32.51**	3.3E-05	4.493998
Within groups	1226.5	16	76.6			
Total	3719.1	17				

As indicated by F values, the mean of removal for bioemulsan (43.0%) is significantly different from the mean of the removal in the experiments with only water (19.5%). This result means that the bioemulsans are effective in the removal of crude oil from contaminated soil. Comparing with ANOVA test, the means of bioemulsan and rhamnolipid (50.8%) treatments the difference of the means did not result significant with a level of significance of 0.05 (F=4.13; p=0.58).

Furthermore, ANOVA test was used for the experiments of bioemulsan and rhamnolipids treatment to individuate the whether soil:washing solution ratio and time of washing, significantly, affect the oil removal. The test showed a positive correlation was found between time of washing with bioemulsan and oil removal.

4. Discussion

The results allow us to evaluate the applicability of SACs produced by *Gordonia* sp. BS29 in environmental remediation techniques for hydrocarbon contaminated soil. Results from bioremediation experiments are very contrasting. Rhamnolipids are known to be effective as enhancers in biodegradation of both aliphatic and aromatic hydrocarbons [1]. However, in our experimental systems rhamnolipids did not succeeded in increasing neither the rate nor the extent of biodegradation for most of the individual contaminants. Actually, cases of inhibitions by biosurfactants are reported as well in the literature and the reasons of these failures have been put forward. The surfactant micelles can provide a barrier between bacteria and the contaminants [14,15]. The possibility for microorganisms to access the micelle-contaminant complex depends on the hydrophobicity of the cell surface [16]. Furthermore the surfactants can inhibit hydrocarbon degradation due to competitive biodegradation [17]. Like rhamnolipids, BS29 bioemulsan did not result in enhancing rate and extent of biodegradation. Since the treated control of the experimentation resulted negative, on the basis of the results, we cannot definitely conclude that BS29 bioemulsan is not effective. Other experimentations are needed in different soils or microbial community to definitely assess its applicability.

On the other hand, results from soil-washing experiments demonstrated that the BS29 bioemulsan effectively removes crude oil from soil. The removal efficiencies are comparable to those of rhamnolipids. Moreover, considering that the bioemulsan was used as crude lyophilized product at the same concentration of rhamnolipids as pure active principle and that for the bioemulsan a positive correlation was found between time of washing and oil removal, there is the potential to further increase the extent of contaminant removal.

Acknowledgements The support by ISPESL (Project: Biosynthesis and characterisation of surfactants for hydrocarbon-contaminated site remediation) is gratefully acknowledged.

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Biosurfactant production by *Chromobacterium prodigiosum*

H. W. C. Araújo[1,2], B. S. O. Ceballos, G. M. Campos-Takaki[2]

[1] Doutoranda, RENORBIO, Universidade Católica de Pernambuco - (UNICAP-PE)
[2] UNICAP, Núcleo de Pesquisas em Ciências Ambientais (NPCIAMB), Recife, PE, Brasil;
[3] Universidade Estadual da Paraíba - UEPB, Departamento de Biologia, Paraíba,Brasil

e-mail- helviaw@yahoo.com.be, bso@superig.com.br, takaki@unicap.com.br

The surfactants constitute an important class of chemical used in industrial sections that act as much dispersion as dissolvable of organic compositions presenting low solubility in water. Now it is growing the interest for biosurfactant, that present several advantages in relation to the synthetic surfactant. This work studied the biosurfactant production for *Chromobacterium prodigiosum* (*Serratia marcencens*) deposited in the bank of cultures of the Nucleus of Researches in Environmental Sciences of UNICAP/PE - Brazil. It was made a factorial experimental planning 2^3 with 4 central points using the corn steep, lactose and wheat maize oil. The experiment was accomplished in Erlenmeyer's containing 100ml of the middle of production and to add 1 ml of the sub-culture, submitted to 150 rpm for a period of 72H to the temperature of 28^{o}C and simultaneously in the same conditions without shake and compared the results. The biosurfactant production was evaluated by the index and emulsification activity and surface tension. The results presented reduction in the surface tension of 25.88(mN/m) when submitted to the shake, the emulsification index was better in the conditions without shake, the emulsification activity obtained significant results in all the conditions in the two experiments.

Keywords Biosurfactant, *Chromobacterium prodigiosum* (*Serratia marcencens*), surface tension

1. Introduction

The industrial process need for surfactants is in constant growth [1] the commercial importance of the surfactants in the environmental area; it is evidenced in the great number of industrial applications. The great majority of surfactants today available it is synthesized starting from derived of the petroleum. However new protection legislations to the environment, as well as the environmental concern among the consumers, they have been taking to the search for natural surfactants as alternative to the existent products [2]. The search for natural surfactants has been subject of great interest of the biotechnology, in function of the need of environmental preservation.

In that context, they stand out the metabolites produced by microorganisms, the calls biosurfactants [3]. The biosurfactants use in the environment is related mainly the bioremediation of derived hydrocarbon of the petroleum in sea atmospheres and in soils [4], has been attracting great attention for they present some advantages front to the chemical surfactants, as low toxicity, biodegradable and ecology acceptability. For the commercial production of the biosurfactant some are being developed strategies so that they can compete with the chemical surfactants [2], the use of low cost substrate is an example, what allows a reduction of more than 50% of the final costs of biosurfactant production [5]. Several bacterium and mushrooms are known for they produce emulsification extra cell, when cultivated in substrate insoluble in water, among the bacterium we can mention: *Pseudomonas aeruginosa, Bacillus subtilis, Rhodococcus sp, Serratia sp* among other as well as species of yeasts as *Candida lipolytica* that also present a great potential in relation to the biosurfactant production, once they are capable to use several hydrocarbon as source of carbon [6]. The biosurfactants are also used in processes of decontamination of terrestrial and aquatic atmospheres, and due to the capacity of degradation of toxicant substances, it has been of great interest in recalcitrant atmospheres [2]. *Chromobacterium prodigiosum (Serratia marcencens)* they are bacilli gram-negatives belonging to the enterobacteriaceae, pieces of furniture with scourges peritriquous, optional aerobic grows well among the temperatures from 37 at 25^{o}C, it produces a pigment denominated prodigiosin. Distributed thoroughly in the nature, those microorganisms are found in the soil, water and plants, as it indicates the name of the family, in the human beings intestinal treatment and you encourage homoeothermic [7].

The objective of that work was to evaluate the production of the biosurfactant for the index and emulsification activity, and surface tension produced by *C. prodigiosum (S. marcencens)* using factorial planning of 2^3 with four central points tends as variables corn steep, lactose and wheat maize oil maintained in orbital shake of 150 rpm to 28^{o}C for a period of 72H, the same conditions they were repeated without agitation to compare the best results in the production of the biosurfactant.

2. Materials and Methods

2.1 Microorganism

The used microorganism was the bacteria *Chromobacterium prodigiosum* (*Serratia marcencens*) belonging to the Bank of Cultures of the Nucleus of Researches in Environmental Sciences (NPCIAMB/PE) of the Catholic University of Pernambuco - Brazil, maintained at 5^{o}C in nutritious agar. The used substrate was the wheat maize oil and the lactose, added the middle of middle of corn steep and Luria Bertani (LB) according to the established conditions in agreement with factorial planning.

2.2 Factorial planning

Initially, it was done a complete factorial planning 2^3 with 4 repetitions in the central point to analyze the main effects and interactions of the variables concentrations of the corn steep, lactose and wheat maize oil about the variable answer surface tension, index and emulsification activity. The analysis of the results was accomplished using the program STATISTIC version 6.0 of Stat soft, it uses.

2.3 Preparation of the sub-culture

The preparation of the sub-culture it was done in an Erlenmeyer flask containing 100ml of half Luria Bertani (LB) and inoculated three competences of the pure culture maintained under orbital shake from 150rpm at 28^{o}C, kindred of obtaining a young culture.

2.4 Production of biosurfactant by *C. prodigiosum (S. marcencens)*

The cultivations for the production of biosurfactant for *C. prodigiosum* (*S. marcencens*), they were accomplished in flasks of Erlenmeyer's of 250ml of capacity containing 100ml of the middle of production in agreement with the factorial planning (Table 1) inoculated by 1ml of the sub-culture. The flasks were maintained by 150rpm, incubated by 72H and temperature of 28ºC the same conditions they were repeated in Erlenmeyer's flasks without shake to compare the best results in the production of the biosurfactant. After this period, aliquots were used to measure surface tension, emulsification index and emulsification activity (U.A.E.) on the cell-free broth obtained centrifuging the cultures at 5000 rpm for 20 minutes. The surface tension was determined with a Tensiometer model Sigma 70 (KSV Instruments LTD - Finland) using the Du Nouy ring method at room temperature. The emulsification index was analyzed according to Cooper and Goldenberg [8], whereby 2 ml of n-hexadecane was added to 2 ml of the cell-free broth in a graduated screw cap test tube and vortexed at high speed for 2 min and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100. Emulsification activity was determined using the method described by Cirigliano and Carman [9]. After 10min of rest, the formed emulsions were removed with the aid of the pipette of Pasteur, placed in a cubete and later read in spectrophotometer in the wavelength to 540nm.

3. Results and Discussions

3.1 Biosurfactant production for *C. prodigiosum (S. marcencens)*

In the area biotechnology, surfactants produced by microorganism they are important, because they present special advantages on the chemical surfactants as low toxicity, biodegrability, production of renewable substrate and stability. The biosurfactant application is wide, as in spills of petroleum, removal of oily dregs of tanks (cleaning) of reserve and to in the production of victuals[10]. The production of the biosurfactant was conducted using a factorial design, as described in Table 1. The numbers from one (1) to eight (8) correspond to the experimental conditions obtained from the combination of the variables tested and the numbers nine (9) to twelve (12) correspond to the conditions of the central point. After 72H of cultivation, a significant reduction of the surface tension of the water 70 mN/m was observed to 25.88 mN/m in the condition 8 with shake, where it happened larger corn steep concentration and wheat maize oil, while in the without shake the smallest tension was observed in the condition 3, where it happened a larger wheat corn steep concentration. Those results were quite promising, considering that factorial planning's accomplished by [11], in the production of the biosurfactant with the strain *Serratia sp* SVGG 16 using the gasoline as substrate obtained a reduction of

34.4 mN/m, after 96 hours of recent cultivate. Study accomplished with the bacteria *Bacillus subtitles* they demonstrated the capacity of this microorganism in the reduction of the surface tension for 27 mN/m [12].

The best result regarding the emulsification activity for emulsions water-n-hexadecane it was obtained in the condition 4 (shake), that was of 5.87 U.A.E. and of 5.71 U.A.E. in the condition 2 without shake. Already for the emulsification index the condition 5 it presented the best result 68.8% for emulsion water-n-hexadecane (Table1). Tests accomplished by GHURYE et al (1994) [13], they demonstrate that the capacity of emulsification of the biosurfactant produced by *Escherichia coli* JM 101 it was not significant, once the formed emulsions presented activity around 0.9 U.A.E. As control of the test of emulsification activity some commercial surfactants were tested and the activities presented values below 2.58 U.A.E, therefore, the activity values and emulsification index obtained in this work they were quite significant.

Table 1: Results of the process fermentation of *C. prodigiosum (S. marcencens)* using factorial planning of 2^3 with 4 repetitions in the period of 72H, tends as variables answers surface tension, activity and emulsification index

Conditions	Lactose	Cornsteep	Maise oil	Surface tension (mN/m)		Activity emulsification U.A.E.		Emulsification index (%)	
				A*	B**	A*	B**	A*	B**
1	0.2	1.0	5.0	33.12	34.60	5.27	5.06	0.0	0.0
2	1.0	1.0	5.0	26.70	37.50	5.50	5.71	0.0	17.2
3	0.2	6.0	5.0	33.35	28.62	4.62	4.81	0.0	28.6
4	1.0	6.0	5.0	26.29	27.80	5.87	4.41	42.8	62.5
5	0.2	1.0	7.5	29.90	49.35	5.55	5.54	0.0	68.8
6	1.0	1.0	7.5	26.50	47.66	4.34	5.47	15.2	34.3
7	0.2	6.0	7.5	34.25	47.68	5.34	3.03	0.0	66.7
8	1.0	6.0	7.5	25.88	29.60	4.42	5.52	0.0	62.8
9	0.6	3.5	6.25	28.84	42.45	3.35	>6.0	0.0	68.6
10	0.6	3.5	6.25	29,61	40.06	3.46	>6.0	0.0	64.3
11	0.6	3.5	6.25	29.53	43.35	3.37	>6.0	0.0	68.6
12	0.6	3.5	6.25	29.13	43.10	3.33	>6.0	0.0	68.8

A*- shake 150rpm, B**- without shake

3.2. Main effects of the variables used on the surface tension of the surfactant produced by *C. prodigiosum* (*S. marcencens*) with 72H of cultivation with and without shake

The employment of the statistical modelling consists of an important tool that can be used to explain the most important influence as well as the interaction so much among the influences of the parameters fermentation involved in the acting of a certain process MYERS AND MONTGOMERY,2002[14]. In this sense, planning's factorial sequence were used as statistical tool to accomplish the process of optimization of the biosurfactant production for *C. prodigiosum (S. marcencens*), using as substrate lactose and wheat maize oil in means of Luria Bertani (LB) and corn steep (industrial residue).

The reduction of the surface tension is used as a primary criterion to select microorganism producing of biosurfactants, although agents emulsification and dispersant don't possess, necessarily, ability in reducing the surface tension. Now several studies are gone back to optimization of the biosurfactants production, using means of low cost culture associated to insoluble substrate [6]. In agreement with the diagram of Pareto the effects standardized for a level of 95% of trust, it can be observed that in the Figure 1A (shake); the lactose presented a significant influence under the statistical point of view acting in the tension surface. The maize oil in spite of not having presented a resulted significant statistic it also acted in the reduction of TS and the corn steep besides not having presented resulted significant statistic it also increased TS.

The combination of the variables lactose and corn steep it also presented significant influence in the reduction of TS, however the interaction between the variables corn steep and maize oil, as well as Lactose and maize oil didn't present significant results under the statistical point of view and they didn't act in the reduction of the surface tension. The Figure 1B, shows that the lactose didn't obtain a resulted significant statistic more it reduced TS, as well as the interactions among lactose-corn steep and wheat germ oil, the corn steep already presented a significant influence under the statistical point of view while the maize oil had an influence of the statistical point of view more it acted increasing TS.

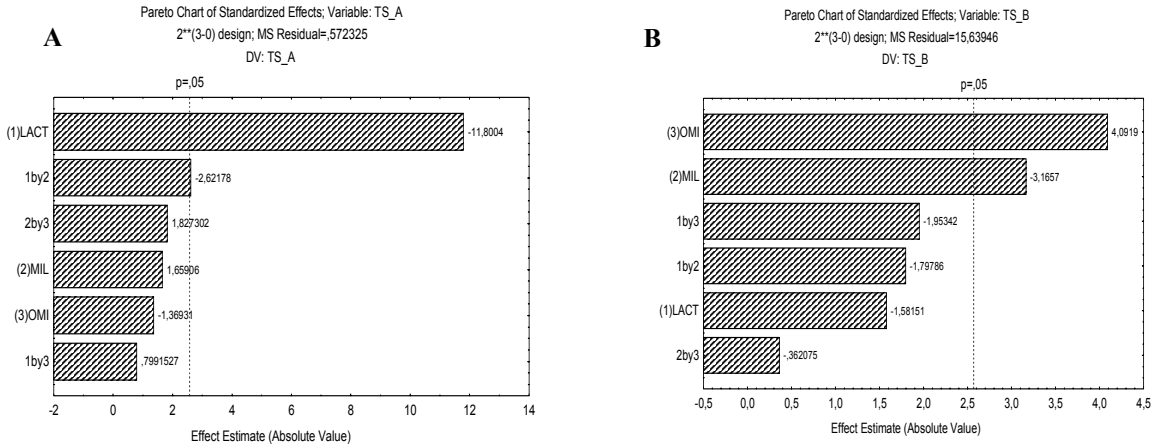

Figure 1 - Diagram of Pareto of the effects of the variables used on the surface tension of the surfactant produced by ***C. prodigiosum*** (***S. marcencens***) with 72H of cultivation (A - Shake; B - Without Shake)

In the condition A and condition B, the variables answer: activity and emulsification index didn't obtain significant result under the statistical point of view, however the interactions lactose-corn steep and with corn steep-corn oil acted increasing the emulsification activity (Fig.2A). The variable lactose even not presenting significant result increased the emulsification activity in shake, as well as the interaction of the same with the wheat germ oil and corn steep (Fig.2B). The variable answer emulsification index, it can be observed in agreement with the Fig. 2C that the variable lactose even not obtaining a significant result acted increasing the emulsification index, already (Fig.2D), the variables lactose, corn steep and oil and corn acted increasing the index of same emulsification doesn't tend influence of the statistical point of view.

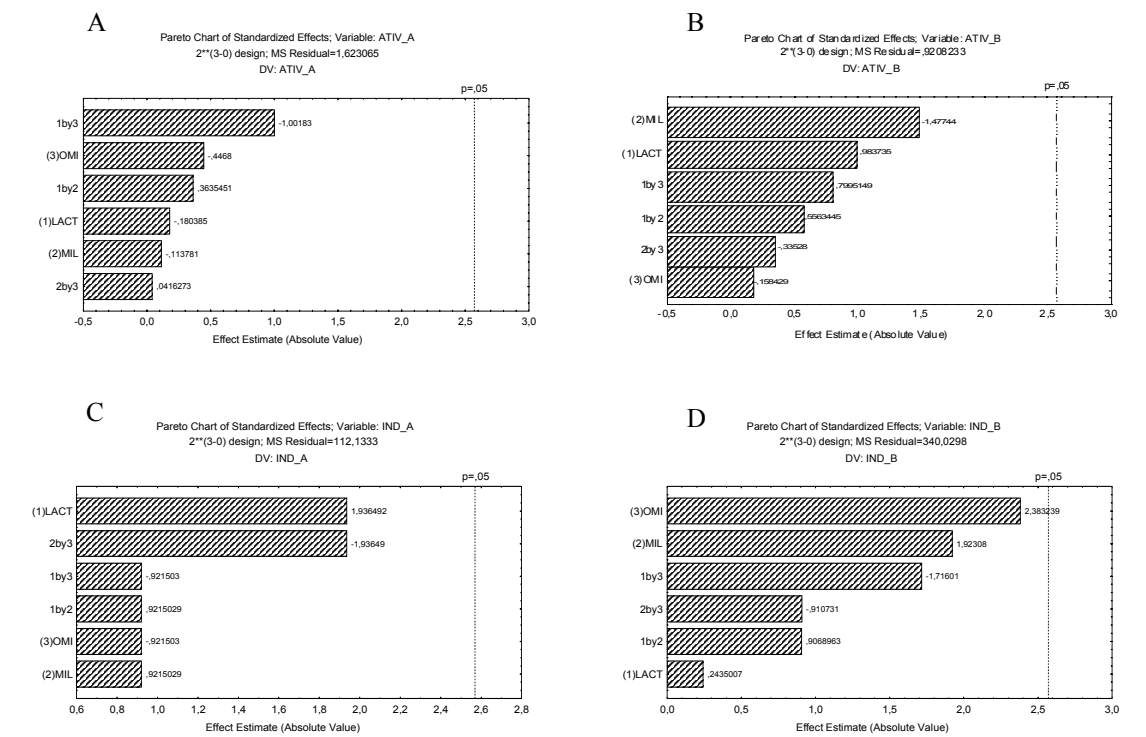

Figure 2 - Diagram of Pareto of the effects of the variables used on the Activity and index of emulsification of the surfactant produced by *C. prodigiosum* (*S. marcencens*) with 72H of cultivation (A-activity emulsification with Shake; B-emulsification activity without Shake, C- emulsification index with Shake, D- emulsification index without Shake)

4. Conclusions

The results obtained significant reduction of the surface tension of the water 70 mN/m to 25.88 mN/m in the condition 8 (shake), in the condition 5(B) reduction of the surface tension was observed where it happened a larger wheat maize oil concentration 28.62 mN/m. The best results in the emulsification activity water-n-hexadecane they were obtained in them condition 4 e 2, with shake and without shake that was of 5.87 U.A.E. and of 5.71 U.A.E. respectively. The emulsification index presented better result in the condition 5 (B) it presented from 68.8% to emulsion water-n-hexadecane. In agreement with the Pareto diagram it can be observed that the lactose presented a significant influence under the statistical point of view acting in the tension surface (TS). The Fig.1B, shows that the lactose didn't obtain a resulted significant statistic more it reduced TS, as well as the interactions among lactose-corn steep and lactose- maize oil. The Fig.2A, B, C and D didn't obtain variables answer for index and activity of emulsification of the statistical point of view.

Acknowledgements The authors are grateful the financial support CAPES, FINEP and CNPq.

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Emulsifiers agents produced by *Candida lipolytica* cultivated in insoluble substrates

R. Diniz Rufino[1,3], J. Moura Luna[2,3], C. B. Barbosa Farias[3], C. A. Buarque Gusmão[3], L. Asfora Sarubbo[3] and G. M. Campos-Takaki[3]

[1] Pós-graduação em Biologia de Fungos, Universidade Federal de Pernambuco, Recife-PE, Brazil
[2] Pós-graduação em Ciências Biológicas, Universidade Federal de Pernambuco, Brazil
[3] Núcleo de Pesquisas em Ciências Ambientais (NPCIAMB), Universidade Católica de Pernambuco, Recife-PE, Brazil

In the present work we described the production of emulsifier agents by the yeast *Candida lipolytica* cultivated in mineral medium supplemented with different substrates, such as petroleum, n-hexadecane, diesel, motor oil and corn oil. The kinetic growth was monitored during 144 hours at 200 rpm and the emulsification activity was determined at the end of cultivation for different hydrocarbon substrates. The results obtained showed that higher emulsification indexes were observed for petroleum and motor oil, in despite of the carbon source utilized for bioemulsifier production. The emulsification capacity of the emulsifying agents was tested in the metabolic broth free of cells under different temperatures and salt concentrations. The bioemulsifiers obtained showed specificity for petroleum and motor oil emulsification. The strong emulsification activity against petroleum and effectiveness at extreme temperatures and salt concentrations indicate that the bioemulsifiers are suitable for use in the petroleum industry.

Keywords bioemulsifiers; *Candida lipolytica*; stability

1. Introduction

Almost all surfactants being currently produced are chemically derived from petroleum. However, these synthetic surfactants are usually toxic themselves and hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment [1].

These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the microbial production of surfactants. Surfactants possess both hydrophilic and hydrophobic structural moieties, which in turn impart many unusual properties, including an ability to lower the surface tension. Biosurfactants are derived from living organisms, mainly microorganisms, and have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and biodegradability [2]. Their environmental uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and in the degradation of hazardous compounds. In the oil industry, they are used in microbial-enhanced oil recovery, in the cleaning of contaminated vessels and to facilitate transportation of heavy crude oil by pipeline [3]. Yeasts are known to produce extracellular emulsifiers when grown on water-immiscible substrates such as alkanes or oils, in order to facilitate their uptake. Among yeasts, *Candida* species have been widely used for hydrocarbon cultivation and production of biosurfactants [4].

The objective of this work was to investigate the production of bioemulsifiers from *Candida lipolytica* using different insoluble substrates aiming the application of these agents in the environment.

2. Materials and methods

2.1 Microorganism and maintenance

The microorganism *Candida lipolytica* UCP 0988 was kindly supplied by the Culture Collection of the Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Brazil. The microorganism was maintained as the anamorph state at 5ºC on Yeast Mold Agar (YMA) slants. Transfers were made to fresh agar slants each month to maintain viability.

2.2 Growth conditions

Cultures were grown in a mineral medium containing NH_4NO_3 0.1%, KH_2PO_4 0.02%, $MgSO_4.7H_2O$ 0.02%, yeast extract 0.1% and 2% of one of the following carbon sources: petroleum and derivates (motor oil, n-

hexadecane and diesel), corn oil and glucose, as the control. The culture liquid pH was adjusted to pH 5.7 with 1 M NaOH solution or 1 M HCl solution. The *C. lipolytica* was grown in solid medium at 27ºC for 24 h; then, a loopful of the culture was transferred to Erlenmeyer flasks of 250 ml capacity containing 50 ml of the liquid medium, Yeast Mold Broth (YMB) and incubated aerobically for 1 day at 27ºC on a rotary shaker at 150 rev/min. The YMB culture contained 10^4 cells/ml and was used to initiate growth in the mineral medium using a 10% v/v inoculum.

2.3 Bioemulsifiers production

The production of the bioemulsifiers were carried out in Erlenmeyer flasks of 500 ml capacity containing 100 ml of the medium supplemented with the carbon source and shaking at 200 rev/min for 144 h at 27ºC. Samples were taken throughout the cultivation period and the following characteristics were determined: yeast biomass, monitored by cells counts on Neubauer Camera and the culture liquid acidity (pH).

2.4 Enzyme determination

The enzymatic qualitative test was cerried out after the production of the enzymes lipase and esterase in the mineral medium containing the specific substrate during 144 hours of cultivation. The supernatants obtained were used for enzymatic determination according to Kitancharoen and Hatai [5], for esterase and [6], for lipase, at 27°C temperature during 48-72 hours in Petri plates.

2.5 Emulsification index

The emulsification index was measured according to the method described by Cooper and Goldenberg [7] whereby 2 ml of hexadecane or other hydrocarbon was added to 2 ml of the cell-free broth in a graduated screwcap test tube and vortexed at high speed for 2 min. Negative control of emulsification was carried out using water and the hydrocarbon substrate. The emulsion stability was determined after 24 h, and was observed during 7 days, and the emulsification index (E) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.6 Stability studies

Stability studies were conducted using the cell-free broth obtained centrifuging the cultures at 5,000 rev/min for 20 min. Four milliliter samples of the cell-free broth were heated at 100ºC, and cooled to room temperature, after which the emulsification activity was measured. The emulsification capacity of cell-free broth was also determined after exposure at lower temperature (4ºC). The effect of NaCl concentrations (2.0, 5.0, and 10.0%) on the emulsification capacity was also determined [8].

3. Results and discussion

3.1 Kinetic growth of *Candida lipolytica*

Previous reports have demonstrated the use of hydrocarbons and the combination of a carbohydrate plus vegetal oil as alternatives for production of bioemulsifiers by yeasts [9, 10, 2, 3].
Bioemulsifiers production was studied in experiments by varying the substrates petroleum and derivates (motor oil, n-hexadecane, diesel,) and corn oil. The changes in cell growth and pH with time are shown in Figure 1.

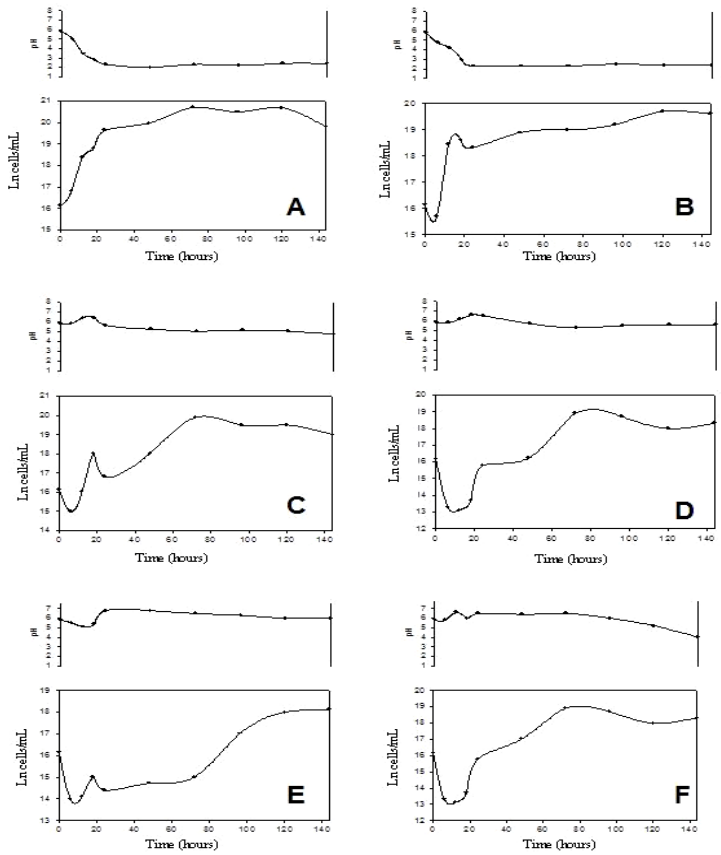

Fig. 1 Growth and pH of *Candida lipolytica* grown in mineral medium supplemented with 2% of: (A) Glucose; (B) Corn oil; (C) Diesel; (D) Petroleum; (E) Motor oil and (F) Hexadecane

The kinetic growth of *C. lipolytica* cultivation in mineral medium containing petroleum or derivates showed that the yeast reached the stationary growth phase after 60 h. For these cultivation conditions, the pH remained around 6 during fermentation, except for the medium containing hexadecane, for which the pH reached values around 4.0. Differently, in the medium supplemented with the vegetal oil (Figure 1B) or glucose (Figure 1A), the microorganism had entered the stationary phase after about 20 hours, while the pH reached values around 2.0. These differences can be explained by the complexity of the fatty acid chains of the petroleum and derivates carbon sources leading to a larger period of time by the microorganism to reach the stationary growth phase.

3.2 Emulsification index

Stabilization of an oil and water emulsion is commonly used as a surface activity indicator. Emulsification activities of the bioemulsifiers produced were measured with various hydrocarbons (Table 1). It could be observed that higher emulsification activities were obtained using petroleum, motor oil and corn oil, despite of the carbon source used for the production of the bioemulsifiers. These results suggest that the activity of the emulsifier depends more than its affinity with hydrocarbon substrates, which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium.

Table 1 Emulsification of hydrocarbons by the cell-free broth of *Candida lipolytica* cultivated in different carbon sources during 144 hours

Hydrocarbons	**Carbon sources / Emulsification (%)**					
	Petroleum	**Motor Oil**	**Diesel**	**Hexadecane**	**Corn Oil**	**Glucose (Control)**
Petroleum	100%	100%	100%	100%	100%	100%
Motor Oil	100%	100%	100%	100%	100%	60%
Diesel	---	---	---	---	---	---
Hexadecane	---	---	---	---	30%	---
Corn Oil	---	---	50%	---	20%	60%

Interestingly, the *Candida lipolytica* cultivated in the soluble substrate glucose (control) produced high values of emulsification activity. These results demonstrate that the synthesis of biosurfactants is not simply a prerequisite one for the degradation of hydrocarbons. It is important to point out that these results are also in accordance with the findings of Sarubbo et al. [4] who showed the production of a bioemulsifier by this same yeast specie by using glucose as the carbon source.

3.3 Effects of temperature and salinity on emulsification capacity

The emulsification indexes of the bioemulsifiers produced were measured at 5, 25, 50 and 100 ºC (Figure 2). The petroleum was the hydrocarbon most emulsified by all the substrates used for fermentation at all the temperatures studied, followed by motor oil. For this last, the temperature of 50 °C did not allow its emulsification by the bioemulsifier produced in the same substrate. The bioemulsifiers produced in the conditions of this work did not show specificity for diesel and hexadecane, once these hydrocarbons were not emulsified in the temperature range tested.

The effect of NaCl addition on the emulsification capacity of the bioemulsifiers produced by *Candida lipolytica* is shown in Figure 3. Petroleum and motor oil were the hydrocarbons more emulsified, although the addition of 10% of the salt inactivated its emulsification by most of the emulsifiers produced. The hexadecane was only effectively emulsified by the bioemulsifier produced with petroleum as carbon source under 2% NaCl, (Figure 3), while only the addition of 10% salt was vantageous for corn oil emulsification by the bioemulsifier produced with petroleum. The addition of NaCl in the concentrations tested did not improve the emulsification of diesel. Most known surfactants are less stable over such salt concentrations [11]. According to [12] salt concentrations of 2–3% are sufficient to inactivate conventional surfactants.

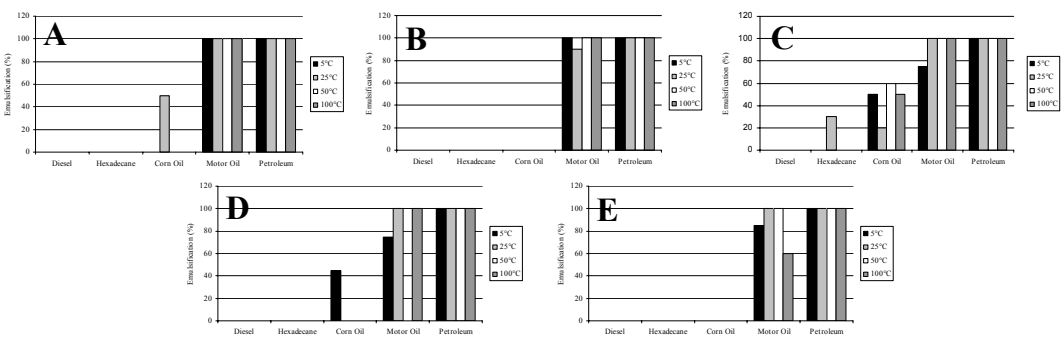

Fig. 2 Effect of temperature on the emulsification capacity of the bioemulsifiers produced by *Candida lipolytica* cultivated in mineral medium containing (A) Petroleum; (B) Motor oil; (C) Diesel; (D) Hexadecane and (E) Corn oil

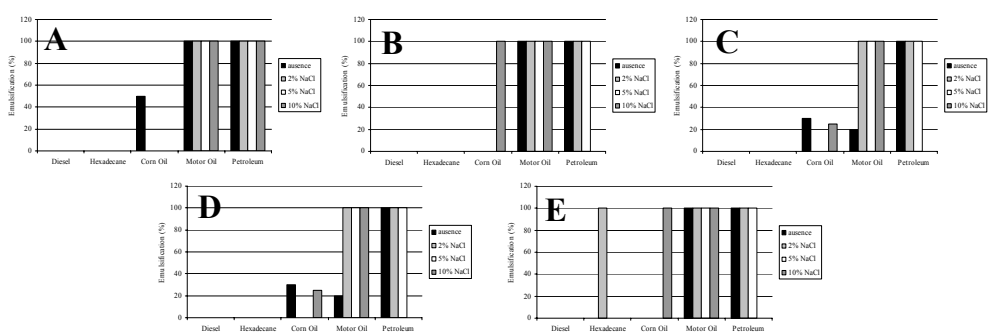

Fig. 3 Effect of NaCl addition on the emulsification capacity of the bioemulsifiers produced by *Candida lipolytica* cultivated in mineral medium containing (A) Petroleum; (B) Motor oil; (C) Diesel; (D) Hexadecane and (E) Corn oil

3.4 Enzymatic activity

The positve tests for detection of lipase and esterase revealed the activity of these enzymes in the utilization of the insoluble substrates used in this work for bioemulsifiers production from *C. lipolytica*.

4. Conclusions

The results obtained in this work revealed the ability of *Candida lipolytica* to produce biosurfactants with emulsification properties from insoluble substrates. The emulsifiers produced showed activity against petroleum and motor oil and stability under a wide temperature range and high salt concentrations. These characteristics show the possible use of these surface agents in the petroleum industry involving environmental applications such as enhanced oil recovery, cleaning of oil reservoirs, reducing oil viscosity for crude oil transportation, and decomposition of spilled oils in soil or marine environments.

Acknowledgement: This work was carried out with the financial support of CAPES/FACEPE, FINEP/CTPETRO, CNPq and Universidade Católica de Pernambuco, Brazil.

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Isolation and screening of surface active compound-producing bacteria on renewable substrates

C. Ruggeri[1], A. Franzetti[*,2], G. Bestetti[2], P. Caredda[1], P. La Colla[1], M. Pintus[1], M.T. Tedde[1], and E. Tamburini[1]

[1] Department of Biomedical Science and Technology, University of Cagliari, Cittadella Universitaria, Monserrato (CA), Italy.
[2] Department of Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, Milano, Italy.

[*]Corresponding author: e-mail: andrea.franzetti@unimib.it, Phone: +39-2-64482927

New bacterial strains were isolated from a site historically contaminated by diesel. The phylogenetic diversity of the isolates was evaluated by ARDRA and 16S rRNA gene sequence analysis. The study was focused on bacterial strains belonging to taxa which have never (or poorly) studied for the production of surface active compounds. 22 strains were screened for the production of both biosurfactants and bioemulsifiers. The use of cheap raw materials will facilitate the future development of economically efficient industrial-scale processes. Thus, the ability of the selected strains to produce surface active compounds on low cost renewable substrates was evaluated. 13 new bioemulsifier- and 8 new biosurfactant-producing bacteria were identified. The majority of the SAC-producing bacteria belongs to Actinobacteria and α-proteobacteria. Among them, the *Gordonia* sp. strain BS29 efficiently produces bioemulsifiers both on soluble and insoluble renewable substrates, such as molasses, plant and waste oils. Furthermore, the strain BS29 produces biosurfactants on a wide range of plant and waste oils.

Keywords surface active compound; biosurfactant; bioemulsifier; renewable substrate

1. Introduction

Surfactants are amphiphilic molecules with both hydrophilic and hydrophobic moieties. The surfactant market now exceeds 7,000 million euro per year. Surfactants find application in almost every industrial sector. Most of them are chemically synthesized by petroleum, not easily biodegradable and toxic to the environment. In addition, their manufacturing processes and by-products are environmentally hazardous [1]. Increased environmental awareness and new regulatory legislation point to the environmental compatibility of surfactants as an important factor in their application.

Prokaryotic and eukaryotic microorganisms synthesize a wide range of structurally different amphiphilic molecules and produce both extracellular and cell-bound compounds. Neu [2] divided microbial surface-active compounds (SACs) into low molecular weight SACs, also termed biosurfactants, and high molecular weight SACs which include amphiphilic and polyphilic polymers. The former lower surface and interfacial tensions, whereas the later are usually more effective in stabilizing oil-in-water emulsions, and thus are known as bioemulsifiers.

When compared to their synthetic counterparts, many microbial SACs show superior properties and advantages, such as high surfactant and emulsifying activities, stability in extreme chemico-physical conditions, low toxicity, high biodegradability, and the ability of being produced from renewable and cheap raw materials [1]. These characteristics result in a greater acceptability, especially in the applications that cause the dispersion of SACs in the environment. The heterogeneity of their structural types and properties results in a broad spectrum of potential applications in oil industry, environmental remediation, agriculture, as well as medicine, cosmetic and food industries [1].

Costs are often the bottleneck of a biotechnological process, and this is also the case for the production of SACs. At present, the potential profit from microbial surfactants is not competitive with that of their chemical counterparts, due to high costs and low yields of production. The costs of microbial metabolites is governed by three basic factors: (*i*) initial raw material costs, (*ii*) availability of suitable and economic production and recovery procedures, (*iii*) product yields by the producer microorganisms. Different strategies have been adopted to make biotechnological processes cost-competitive: (*i*) use of cheap substrates or wastes (*ii*) development of efficient bioprocesses, including optimisation of fermentative conditions and recovery processes, (*iii*) selection of over-producing strains [3]. However, research on SACs, particularly related to the development of cost-effective production processes, has been confined mostly to few well-characterised genera (*Acinetobacter*, *Bacillus*, *Pseudomonas* and *Candida*) and, consequently, a limited spectrum of products has been evaluated for commercial applicability [3]. Furthermore, only few studies have concerned the phylogenetic diversity of SAC-producing microorganisms, yet [4,5,6].

The aim of this work was to analyse the phylogenetic diversity of SAC-producing microorganisms isolated from a site historically contaminated by diesel. The study was focused on bacterial strains belonging to taxa which have never (or poorly) studied for the production of bioemulsifiers and biosurfactants. The search of bacterial strains able to synthesise SACs on low cost renewable substrates was carried out. The use of cheap raw materials will facilitate the future development of economically efficient industrial-scale biotechnological processes.

2. Materials and Methods

2.1 Sampling, enrichment, isolation and 16S rRNA gene sequence analysis

Samples were collected from a site historically contaminated by diesel. 4 samples of non-contaminated vadose zone soil (≈ 2 m) and 5 samples of hydrocarbon-contaminated capillary fringe soil (≈ 5 m) were obtained using a piston corer. NAPL was collected from the groundwater. An aliquot of the NALP was sterilized by filtration through 0.2 μm filters and used as carbon and energy source (20 g/l) during enrichment and isolation procedures.

The extraction of bacteria from soil was performed by suspending 2.0 g of soil sample in 18 ml of NaCl solution (0.85 g/l) and vortexing for 2 min. The hydrocarbon-degrading bacteria were enumerated by MPN procedure [7]. Enrichment cultures were prepared using soil samples as *inoculum* and the sterile NAPL as carbon source. Alternatively, the enrichment cultures were prepared using the non-sterile NAPL both as *inoculum* and carbon source. The most diluted MPN cultures that exhibited growth or aliquots of enrichment cultures were spread onto BH-agar plates supplemented with sterile NAPL. Colonies were obtained in pure culture by repeated streaks on the same solid medium.

DNA purification, 16S ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequence analysis were carried out as previously described [8,9].

2.2 High throughput screening of potential SAC-producing strains

M1 medium composition was the following (per litre): K_2HPO_4 1.32 g, KH_2PO_4 1 g, NH_4Cl 0.81 g, $NaNO_3$ 0.84 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4$ 0.20 g, $CaCl_2$ 0.02 g. Each different carbon source was filter sterilised and supplied at an initial concentration of 20.0 g/l. The cultures were prepared in 96-well 2 ml culture plate (SIGMA) containing 1 ml M1 medium. Bacteria were inoculated to an initial optical density at 600 nm (OD_{600}) of 0.050. After seven days at 30 °C, the bacterial growth was evaluated spectrophotometrically. Cultures were centrifuge for 10 min at 6.000 rpm. Oil spreading test (OST) and small scale emulsification activity test (EA) were performed on the culture supernatants.

2.3 Evaluation of renewable substrates as raw materials for SAC production

The cultures were prepared in 100 ml Erlenmeyer flasks containing 20 ml M1 medium and inoculated to an initial OD_{600} of 0.050. Each different carbon and energy source was supplied at an initial concentration of 20.0 g/l. Cultures were grown at 30°C in a rotary shaker at 250 rpm. After seven days, emulsification activity, surface tension and critical micelle dilution (CMD) were determined. The measures of bacterial growth on different carbon and energy sources were performed as follow: cell collection by filtration through 0.2 μm filters, detachment of cells from filters by vigorously shaking for 5 min, suspension on saline solution and determination of OD_{600}. All determinations were performed at least in duplicate.

2.4 Determination of SAC activities

OST was performed according to Morikawa et al. [10]. CMD was determined by dilution method as previously described by Pagilla et al. [11]. Analyses were carried out with Wilhelmy plate method using a 3S tensiometer (QBX, France). All determinations were performed at least in duplicate.

During the high throughput screening of potential SAC-producing strains, a small scale emulsification assay was carried out using 500 μl of supernatant and 250 μl of *n*-hexadecane in a 2.0 ml tube. The sample was vortexed 2 min and the mixture was allowed to settle for 24 h. Samples producing a stable emulsion were scored as positive.

During evaluation of renewable substrates for SAC production, the emulsification assay was performed as previously described [9]. The emulsification activity (E24%) is given as percentage of middle emulsion phase normalized to the total volume [12]. All determinations were performed at least in duplicate.

3. Results and Discussion

3.1 Isolation and phylogenetic analysis of bacterial strains

A total of 52 bacterial strains were enriched and isolated in pure cultures from non-contaminated vadose soil, hydrocarbon-contaminated capillary fringe soil and NAPL samples using the sterilized NAPL as selective carbon source (Table 1).

The genetic diversity of the isolates was investigated by 16S ribosomal DNA restriction analysis (ARDRA), a method often generating species-specific restriction groups. DNA was extracted from all the isolates and the 16S rRNA gene was amplified. ARDRA was performed using *Alu* I restriction enzyme. 19 different ARDRA haplotypes were recognised by comparing the restriction patterns of the isolates. Partial 16S rRNA gene sequence was determined for at least a strain from each ARDRA haplotype and compared with the prokaryotic small subunit rRNA sequence database of the Ribosomal Database Project II. Results are shown in Table 1.

Table 1 Phylogenetic analysis of the bacterial strains isolated from non-contaminated vadose zone soil (VZ), diesel-contaminated capillary fringe soil (CF) and non-aqueous phase liquid (NAPL) samples, and evaluation of biosurfactant and bioemulsifier production in the culture supernatants by OST and EA, respectively. [a] Biomass increase was not detected on the tested carbon sources. [b] Most diluted MPN cultures that exhibited growth.

Taxonomic position	Strain	Origin	Selection procedure	ARDRA Haplotype (no. strains)	Nearest relative among species type strains (T)	Identity (%)	OST	EA
Actinobacteria	BSN1	VZ0	MPN (10^{-1}) [b]	I (4)	*Microbacterium phyllosphaerae*^{T}	97.6	-	+
	BSN27C	VZ0	MPN (10^{-1}) [b]	II (2)	*Arthrobacter pascens*^{T}	99.7	-	+
	BSNC10A	VZ4	MPN (10^{-3})	III (2)	*Cellulomonas cellasea*^{T}	99.8	-	-
	BSN30C	VZ2	MPN (10^{-1}) [b]	IV (1)	*Pseudonocardia halophobica*^{T}	96.8	+	+
α-proteobacteria	BSN3	VZ0	MPN (10^{-1}) [b]	V (4)	*Mesorhizobium amorphae*^{T}	96.8	-	+
	BSN13	VZ2	MPN (10^{-1}) [b]	VI (2)	*Inquilinus limosus*^{T}	99.1	-	+
	BSN30A	VZ2	MPN (10^{-1}) [b]	VII (1)	*Afipia broomeae*^{T}	99.5	-	+
	BSN1B	VZ3	MPN (10^{-3}) [b]	VII (1)	*Afipia broomeae*^{T}	99.7	+	+
	BSN5B	VZ4	MPN (10^{-3}) [b]	VIII (2)	*Bosea eneae*^{T}	99.8	+	-
β-proteobacteria	BSN15B	VZ2	MPN (10^{-1}) [b]	IX (1)	*Cupriavidus necator*^{T}	100.0	-	+
Actinobacteria	BS21B	CF0	MPN (10^{-1}) [b]	X (5)	*Arthrobacter globiformis*^{T}	98.7	-	+
	BS25	CF4	MPN (10^{-4}) [b]	XI (4)	*Gordonia terrae*^{T}	99.8	+	+
	BS27A	CF2	Enrichment	XII (2)	*Micrococcus luteus*^{T}	99.8	-	-
	BS29	CF2	Enrichment	XI (1)	*Gordonia terrae*^{T}	99.8	+	+
	BS32	CF2	Enrichment	XIII (1)	*Rhodococcus erythropolis*^{T}	100.0	+	-
α-proteobacteria	BS1	CF3	MPN (10^{-2}) [b]	XIV (5)	*Xanthobacter flavus*^{T}	99.6	ND[a]	ND[a]
	BS11A	CF5	MPN (10^{-2}) [b]	XV(5)	*Xanthobacter autotrophicus*^{T}	99.8	ND[a]	ND[a]
	BS16B	CF5	MPN (10^{-2}) [b]	XIV (2)	*Xanthobacter flavus*^{T}	100.0	ND[a]	ND[a]
	BS9Y	CF5	MPN (10^{-2}) [b]	XVI (2)	*Bosea minatitlanensis*^{T}	98.4	+	-
	BS18B	CF5	MPN (10^{-2}) [b]	XVII (2)	*Rhizobium daejeonense*^{T}	97.2	-	-
	BS10B	CF2	Enrichment	XVIII (1)	*Rhizobium giardinii*^{T}	100.0	-	+
Actinobacteria	M22	NALP1	Enrichment	IX (2)	*Gordonia amicalis*^{T}	100.0	+	+

3.2 Screening of potential SAC-producing strains

Methods allowing the positive selection of SAC-producing microorganisms are not currently available. Thus, each isolates was screened for its ability to produce biosurfactants and bioemulsifers using a high-throughput technique. Medium composition (particularly the type of carbon source) influences whether or not a strain produces SACs as well as the type and amount of the produced SACs. Thus, both water-soluble and water-insoluble substrates were used for SAC production. Furthermore, both pure compounds and complex renewable mixtures were included among the substrates: glucose, *n*-hexadecane, rapeseed oil and sugar-beet molasses.

After seven days incubation, biosurfactant production was semiquantitatively assayed in the culture supernatants by OST. The results were classified as positive when values grater than 0.5 cm were obtained [13].

Bioemulsifier production was qualitatively tested by evaluating the ability of culture supernatants to produce a stable oil-water emulsion (Table 1). 8 new biosurfactant- and 13 bioemulsifier-producing strains were identified. The majority of the SAC-producing strains were assigned to Actinobacteria (8 strains) or α-proteobacteria (7 strains).

The majority of the producers synthesised bioemulsifiers on molasses. Biosurfactant production in Actinobacteria (*Rhodococcus*, *Gordonia* and *Pseudonocardia* genera) occurred only on insoluble substrates whereas *Bosea* spp. strains BS9Y and BSNC5B produced SACs, able to reduce the surface tension, only on molasses.

3.3 Development of a cost-efficient process for SAC production by *Gordonia* sp. strain BS29

It has been estimated that raw materials account for 10 to 30% of the total production costs in most biotechnological processes [3]. A strategy to reduce costs of microbial metabolites is the use of low-cost raw renewable materials.

We recently characterised the production of SACs and their role in the access to hydrocarbons in *Gordonia* strains [9]. Particularly, *Gordonia* sp. strain BS29 has been extensively characterised. The strain produces at least two different types of SACs: extracellular bioemulsan(s), able to produce stable emulsions but not reduce surface tension, and cell-bound glycolipid biosurfactant(s), able to reduce surface tension. In this work, a variety of cheap renewable substrates, including plant-polysaccharides, agro-industrial wastes (sugar-beet molasses), edible and non-edible plant-derived oils, and waste frying oil, was tested as substrates for SAC production by *Gordonia* sp. strain BS29 (Table 2).

The strain BS29 is able to grow efficiently on molasses, a wide spectrum of plant-oils, and on waste frying oil from food industry. However, it did not show a significant biomass increase on plant-polysaccharides (starch, carbossimethyl cellulose and pectin) after seven days.

Gordonia sp. strain BS29 produces bioemulsifier(s) when grown on soluble and insoluble renewable substrates. High emulsification activity was found on molasses, a wide spectrum of plant-oils, and on waste frying oil (Table 2). Biosurfactant production was found only on insoluble renewable substrates. The biosurfactant(s) produced on a wide spectrum of edible plant-oils and on waste frying oil from food industry showed the same effectiveness (Table 2). Furthermore, the efficiency of biosurfactant production, as determined by CMD measure, was higher on low-cost plant oils and waste frying oil (41 x CMC) than on *n*-hexadecane (26 x CMC).

Table 2 Emulsification activity (E24%), surface tension and critical micelle dilution (CMD) of whole culture broths (C) and cell-free culture filtrates (F) of *Gordonia* sp. strain BS29 grown on different renewable substrates. Control pure carbon sources: potassium citrate and *n*-hexadecane. Values are means based on two separate experiments with two independent measurements each.

	E24%		Surface tension	CMD
Substrate 20 g/l	C	F	C	C
Potassium citrate	73.3	35	43.70	<1
n-Hexadecane	39.6	44	30.00	26
Sugar-beet molasses	8.3	31.7	53.75	<1
Corn oil	30.8	31.8	28.90	41
Palm frying oil	16.9	35.6	28.07	41
Peanut oil	41.6	28.3	28.72	41
Rapeseed oil	21.7	28.3	30.00	41
Soybean oil	36.7	35.8	32.77	21
Sunflower oil	47.1	30.1	32.39	41
Castor oil	0	10.7	33.79	14
Linseed oil	25	31.4	39.48	<11
Waste frying oil	26.7	33.3	29.02	41

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Production of a bioemulsifier by *Candida glabrata* isolated from mangrove

J. Moura Luna[1,3], R. Diniz Rufino [2,3], C. B. Barbosa Farias [3], C A. Buarque Gusmão[3], L. Asfora Sarubbo[*,3] and G. M. Campos-Takaki[3]

[1] Pós-graduação em Ciências Biológicas, Universidade Federal de Pernambuco, Brazil
[2] Pós-graduação em Biologia de Fungos, Universidade Federal de Pernambuco, Recife-PE, Brazil
[3] Núcleo de Pesquisas em Ciências Ambientais (NPCIAMB), Universidade Católica de Pernambuco, Recife-PE, Brazil

[*]Corresponding author: e-mail: takaki@unicap.br, Phone: +55 81 21194017

The aim of this work was to study the production of emulsifiers by a *Candida glabrata* isolated from mangrove. Fermentations were conducted in mineral medium supplemented with motor oil or vegetal oil refinery residue as substrates. The influence of substrate concentration, inoculum size and presence of yeast extract were evaluated. The kinetic growth was monitored during 144 hours and the emulsification activity was determined in the end of cultivation for different substrates. From the determination of emulsification activity, the medium formulated with 2.5% refinery residue in the presence of 0.1% yeast extract was selected for bioemulsifier production. The emulsions were stable at a wide temperature range, under different NaCl concentrations and for a long period of time. The isolation of the biosurfactant permitted an increase of almost 300% in the emulsification activity and a yield of 2.5g/l of isolated product. The results obtained showed the ability of the yeast in growing in insoluble cheap substrates and to produce emulsifying agents with potential of application in the petroleum industry.

Keywords bioemulsifiers; *Candida glabrata*; stability

1. Introduction

Biosurfactants are derived from living organisms, mainly microorganisms, and have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity and biodegradability. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries. They can also be used in the bioremediation of soil or sand or in the cleanup of hydrocarbon contamination in groundwater [1]. Even though interest in biosurfactants is steadily increasing, these compounds still do not get compete economically with synthetic surfactants. To reduce production costs, different routes could be could be investigated such as the increase of yields and product accumulation; the development of economical engineering process, and the use of cost free or cost-credit feedstock for micro-organisms growth and surfactant production [2]. Agrorefinery residues with high contents of carbohydrates or lipids could in principle be used as substrates for biosurfactant production [3]. However, few attempts at using wastes for biosurfactants production and only few types of biosurfactants produced from wastes have been reported so far. In this study, the production of biosurfactants with emulsification properties by *Candida glabrata* isolated from mangrove sediments was investigated by using low-cost substrates.

2. Materials and methods

2.1 Microrganism and inoculum preparation

Candida glabrata UCP 1002 was kindly supplied from the culture collection of the Catholic University Pernambuco (UNICAP), Brazil. The yeast was maintained at 5°C on Yeast Mold Agar (YMA) slants. For inoculum preparation, a loopful of the cream coloured culture was transferred to Erlenmeyer flasks of 250 ml containing 50 ml of the liquid medium Yeast Mold Broth (CYM) and incubated aerobically for one day at 27°C on a rotary shaker (150rpm).

2.2 Cultural conditions and bioemulsifiers production

The production medium was composed of the following components: NH_4NO_3 (0.1%), KH_2PO_4 (0.02%), $MgSO_4.7H_2O$ (0.02%) and yeast extract, motor oil and vegetal oil refinery residue according to Table 1. 500-ml Erlenmeyer's flasks were filled with 100 ml of culture liquid and sterilized at 121°C for 20 min. The inoculum was introduced in the amount of 10^4 or 10^5 cells/ml of the 24 h culture grown on CYM. Yeast culture was carried out at 27 °C with shaking at 200 rpm for 144 h in a New Brunswick G-25 shaker. Samples were taken periodically until the end of cultivation period (144 hours), being used for the following determinations: yeast biomass, monitored by cells counts on Neubauer Camera and culture liquid acidity (pH). Experiments were done in duplicate and results reported are the average from three independent experiments.

Table 1 Culture conditions of *C. glabrata* UCP1002

Substrates	Concentration (%)	Productiom medium	Inoculum size (cells/mL)
Refinery residue	1.0	Mineral / Mineral with yeast extract	10^4 / 10^5
Refinery residue	2.5	Mineral / Mineral with yeast extract	10^4 / 10^5
Motor oil	1.0	Mineral / Mineral with yeast extract	10^4 / 10^5
Motor oil	0.5	Mineral / with yeast extract	10^4 / 10^5

2.3 Emulsification index

The emulsification index was measured after 144 hours according to Cooper and Goldenberg [4] where y 2 ml diesel, motor oil, corn oil or other hydrocarbon was added to 2 ml of the cell-free broth in a graduated screw cap test tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index (E) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.4 Determination of the stability of the bioemulsifier

Stability studies were done using the cell-free broth obtained centrifuging the cultures at 5000 rpm for 20 minutes. Samples were maintained at 5, 25 and 100ºC during 15 minutes and cooled to room temperature, after which the emulsification index was measured. The effect of NaCl on the emulsification capacity was determined for concentrations varying from 2.5 to 10%, while the influence of time was observed during 7 days.

2.5 Isolation of biosurfactant

The 144-h culture was filtered through Whatman no. 1 filter paper and centrifuged at 10000 x g for 15 minutes. The cell-free broth was concentrated (500 ml) by freeze drying and extracted three times with chloroform (1:1, by vol.) in a separatory funnel at 28°C [5].

3. Results and discussion

3.1 Kinetic growth of *Candida glabrata*

Bioemulsifier production was first studied in experiments by varying the initials concentrations of the substrates refinery residue and motor oil, presence the yeast extract and size of inoculum as showed in Table 1. The change in biomass and pH of the cultivations with time are shown in Figures 1 and 2. The kinetic growth curves were similar and the stationary growth phase had been reached after 24 hours for both substrates and cultivation conditions. Regarding the supplementation of yeast extract, its presence promoted an increase of the pH (Figure 1B, 1D, 1F, 1H and Figure 2B, 2D, 2F and 2H), while for cultivations without it the pH curves had shown a relative decrease (Figures 1A,1C, 1E, 1G and Figure 2A, 2C, 2E, 2G).

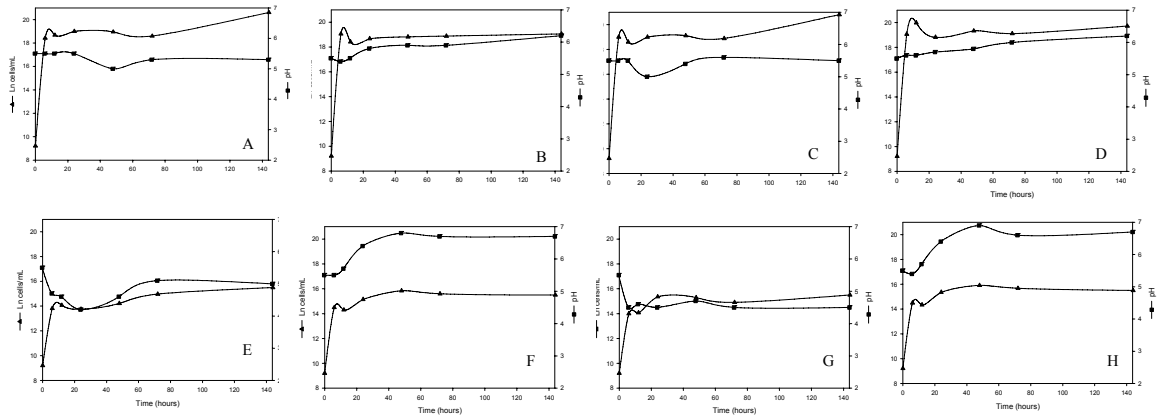

Fig. 1 Time courses of growth and pH of *Candida glabrata* (inoculum size 10^4 cells/ml) cultivated in mineral medium supplemented with: (A) 1% refinery residue; (B) 1% refinery residue and 0.1 % yeast extract; (C) 2.5% refinery residue; (D) 2.5% refinery residue and 0.1% yeast extract; (E) 0.5% motor oil; (F) 0.5% motor oil and 0.1% yeast extract; (G) 1.0 % motor oil; (H) 1.0 % motor oil and 0.1% yeast extract

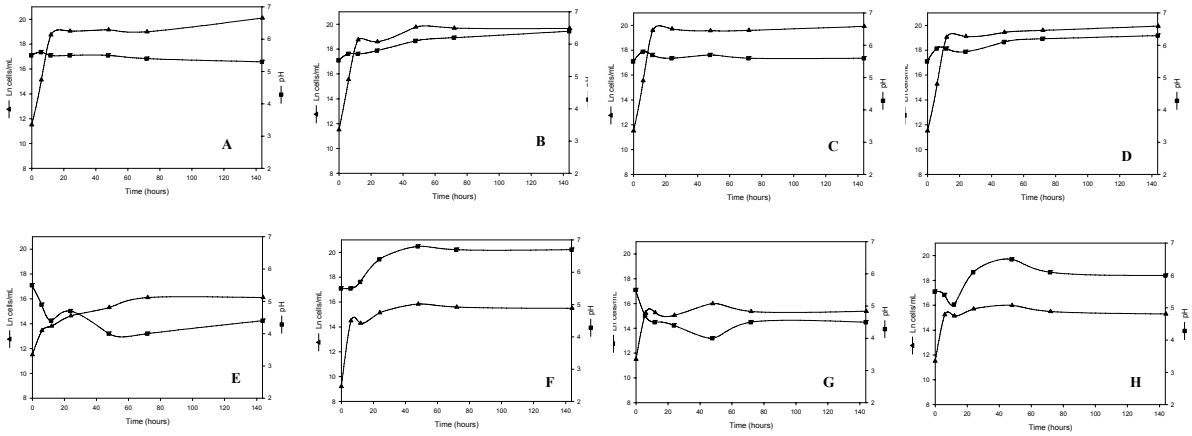

Fig. 2 Time courses of growth and pH of *Candida glabrata* (inoculum size 10^5 cells/ml) cultivated in mineral medium supplemented with: (A) 1% refinery residue; (B) 1% refinery residue and 0.1% yeast extract; (C) 2.5% refinery residue; (D) 2.5% refinery residue and 0.1%yeast extract; (E) 0.5% motor oil; (F) 0.5% motor oil and 0.1% yeast extract; (G) 1.0 % motor oil; (H) 1.0 % motor oil and 0.1%yeast extract

3.2 Emulsification index

Emulsification activities of the bioemulsifiers produced were measured with various water-immiscible substrates (Figures 3-6). Highest emulsification values were obtained using motor oil and corn oil. Diesel was not emulsified effectively. These results indicate that the bioemulsifiers produced under the conditions of this study have high-emulsification specificity toward motor oil and corn oil. Similar results were obtained by Abu-Ruwaida et al., [6] for bacterium identified as *Rhodococus* cultivated on hydrocarbon. These findings suggest that the emulsifier's activity depends on its affinity for hydrocarbon substrates, which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium.

3.3 Properties of the selected bioemulsifier

The initial results obtained permitted to select three emulsifiers which were tested against different substrates, as shown in Figure 7. Between these, the bioemulsifier produced in mineral medium upplemented with 2.5% refinery residue and yeast extract, for the inoculum size of 10^4 cells/mL, was able to emulsify most of the substrates tested and was then chose for further studies.

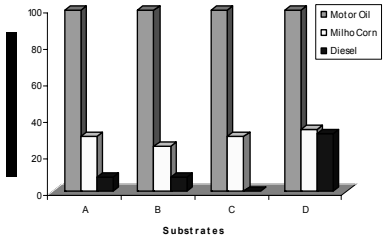

Fig. 3 Emulsification capacity of the cell-free broth of *Candida glabrata* (inoculum size 10^4 cells/ml) grown on mineral medium supplemented with: (A) 1.0% refinery residue; (B) 1.0% refinery residue and 0.1% yeast extract; (C) 2.5% refinery residue; (D) 2.5% refinery residue and 0.1% yeast extract

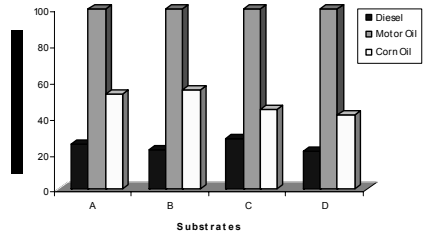

Fig. 4 Emulsification capacity of the cell-free broth of *Candida glabrata* (inoculum size 10^5 cells/ml) grown on mineral medium supplemented with: (A) 1.0% refinery residue; (B) 1.0% refinery residue and 0.1% yeast extract; (C) 2.5% refinery residue; (D) 2.5% refinery residue and 0.1% yeast extract

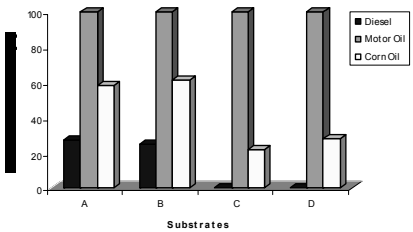

Fig. 5 Emulsification capacity of the cell-free broth of *Candida glabrata* (inoculum size 10^5 cells/ml) grown on mineral medium supplemented with: (A) 1.0% motor oil (B) 1.0% motor oil and 0.1% yeast extract; (C) 2.5% motor oil; (D) 2.5% motor oil and 0.1% yeast extract

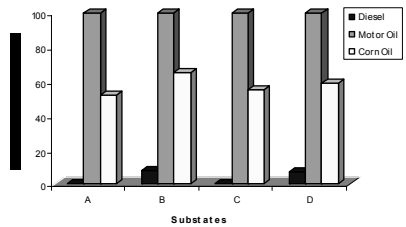

Fig. 6 Emulsification capacity of the cell-free broth of *Candida glabrata* (inoculum size 10^4 cells/ml) grown on mineral medium supplemented with: (A) 1.0% motor oil; (B) 1.0% motor oil and 0.1% yeast extract; (C) 2.5% motor oil; (D) 2.5% motor oil and 0.1% yeast extract

Figure 8 shows the influence of time on emulsification capacity of the cell-free broth containing the bioemulsifier previously selected. Most emulsions formed showed stability during more than a week. Emulsions remained stable for extended periods (>2 months), except the ones formed with kerosene and hexadecane, for which stability was only maintained during 48 hours.

Regarding the influence of the temperature on the emulsification index of the cell-free broth, it was observed that the same stable front stayed to the studied temperatures (Figure 09). Similar behaviors regarding stability were also observed for the biosurfactants produced by *Bacillus subtilis* [7] and by *Nocardia* sp. L-417 [8] when the cell-free broths were heated at 100°C. Liposan from *Candida lipolytica* found to be relatively stable between 30 and 90°C, but lost 60% of its activity after boiling for 1 h [5].

The emulsification capacity of the cell-free broth showed to be little influenced by the presence of NaCl under the concentrations of salt added and the percentiles were specific for each substrate (Figure 10). Studies accomplished by Desai & Banat [9], determined that concentrations above 2% of NaCl are enough for inactive a synthetic surfactant. Reductions were also observed in the emulsification activity of the surfactant produced by *C. lipolytica* cultivated in n-hexadecane [5].

3.4 Bioemulsifier isolation

The examined agent was partially purified from the culture filtrate of *Candida glabrata* cultived in 2.5% refinery residue and 0.1% yeast extract. The precipitate collected in the aqueous phase recovered 300% of the emulsification activity of Corn oil. The average yield of precipitate in the aqueous phase was approximately 2.5g/l. Bioemulsifier production by yeast *Candida utilis* varied from 0.26 to 0.93 g/l and depended on process conditions [10], while the extracellular emulsifying agent from *Curvularia lunata* yielded 2.6 g/l [11].

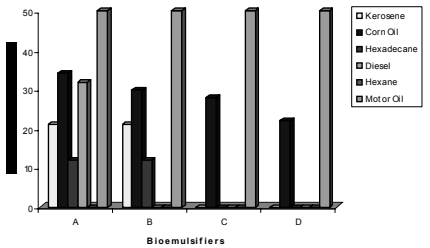

Fig. 7 Emulsification capacity of the cell-free broth of *Candida glabrata* (inoculum size 10^4 cells/ml) grown on mineral medium supplemented with: (A) 2.5% refinery residue and 0.1% yeast extract; (B) 2.5% refinery residue; (C) 1.0% motor oil and 0.1% yeast extract; (D) 1.0% motor oil

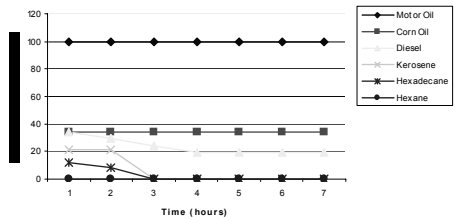

Fig. 8 Influence of time on emulsification capacity of the cell-free broth of *Candida glabrata* grown in mineral medium with 2.5% refinery residue and 0.1 % yeast extract

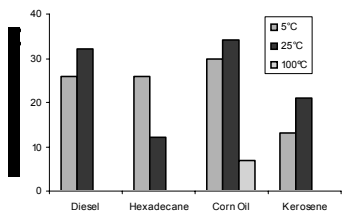

Fig. 9 Influence temperature on emulsification capacity of the cell-free broth of *Candida glabrata* grown on mineral medium supplemented with 2.5% refinery residue and 0.1% yeast extract

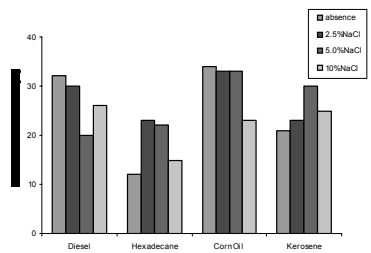

Fig. 10 Effect of different sodium chloride concentrations on the emulsification capacity of the cell-free broth of *Candida glabrata* grown on mineral medium supplemented with 2.5% refinery residue and 0.1% yeast extract

4. Conclusions

The results obtained in this work show that *Candida glabrata* represents a valuable source of new compounds with surface-active properties, with potential of applications in different industries. Work is in progress to improve the production process of these compounds by using industrial wastes as substrates.

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Strategies of Optimization of Bioemulsifier Production by *Candida lipolytica* Using Semidefined Medium

Mabel H. Vance-Harrop[1]; B. Barros Neto[2]; N. B. Gusmão[3];M. C. Ferreira[4]; G. M. Campos-Takaki[5,*]

[1] Doutorado em Biologia de Fungos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brazil; [1] LANAGRO-PE, Ministério da Agricultura, Pecuária e do Abastecimento.
[2] Departamento de Química Fundamental,
[3] Departamento de Antibióticos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brazil;
[4] Mestrado em Desenvolvimento de Processos Ambientais-UNICAP;
[5] Núcleo de Pesquisas em Ciências Ambientais, Universidade Católica de Pernambuco, 50050-590 Recife-Pernambuco, Brazil

[*]Corresponding author: Phone number: +558121194017; Fax:+55-81-211940432. E-mail address: takaki@unicap.br; galba_takaki@yahoo.com.br

The composition of culture media for biosurfactant production in batch cultures of *Candida lipolytica* by a fractional factorial design has been studied. A set of 16 experiments with two central points in duplicate, utilizing five factors (yeast extract, urea, ammonium sulfate, potassium phosphate and babassu oil) at two levels was statistically combined. The analysis of data from this experiment shows a negative effect of the variable ammonium sulfate. Two more factorial design were performed to determine the effect of the lack of ammonium sulfate and urea. The results show that all the factors studied were found to influence biosurfactant production, and that best conditions for higher biosurfactant synthesis is obtained when yeast extract, urea, and potassium phosphate are at a high level +1 concentration. However, with ammonium sulfate at a lower level and babassu oil, the level is independent of the response (biosurfactant production). These results indicated the importance of this study in obtaining maximal production of biosurfactant from *Candida lipolytica* so that this compounds can be used for many purposes.

Keywords Bioemulsifier, Sea water, Medium optimization, Babassu oil, *Candida lipolytica.*

1. Introduction

Surfactants are those chemicals, which are produced by microorganisms but whose amphipatic molecules have both clearly defined hydrophobic and hydrophilic groups. These moieties partition preferentially at the interface between fluid phases with different degrees of polarity, such as oil-water or air-water interfaces [1]. These compounds have the property of reducing surface and interfacial tension in both aqueous solutions and hydrocarbon mixtures, and thereby have potential applications in the recovery of oil, and the pharmaceutical and food. Most of the commercially available surfactants are chemical surfactants, mainly petroleum-derived. However, rapid advances in biotechnology and increased environmental awareness on the part of consumers, combined with expected new legislation in many countries, has provided further impetus for serious consideration of biological surfactants as possible alternatives to synthetic ones [2]. Experimental design techniques are very useful tools for this purpose, providing evidence of the relative influence of various factors studied and determining the optimal concentration calculated for a given target such as maximal growth or maximal metabolite production. In a previous study of biosurfactant production from *C. lipolytica*, this yeast demonstrated the ability to produce molecules with surfactant properties in culture media containing natural seawater and distilled water in the liquid phase [3]. The purpose of this was to determine the best concentrations in a semi-defined medium for the growth and biosurfactant production by the yeast *C. lipolytica.*

2. Materials and methods

2.1 Microorganism

Candida lipolytica- Harrison (Diddens & Lodder) IA 1055, belonging to the culture collection of the Departamento de Antibióticos - Universidade Federal de Pernambuco, was used in this study. The strain was grown on a Yeast Mold Agar (YMA) medium and was maintained at 4°C, at the Núcleo de Pesquisas em Ciências Ambientais – UNICAP (UCP 00065).

2.2 Culture conditions

The media used for the experiments consisted of the following: yeast extract, ammonium sulfate, urea, potassium phosphate, and babassu oil. Distilled water and natural seawater were added at the liquid phase. The inocula were prepared in an Erlenmeyer flask containing 50 ml of Yeast Mold Broth (YMB) and were inoculated using a microbial loop, incubated in an orbital shaker at 250 rpm and 28°C for 24h. The media compounds concentrations for values (-1) were as follows: yeast extract, 1,0 gl^{-1}, ammonium sulfate, 1,0 gl^{-1}urea, 2,5 gl^{-1}, potassium phosphate, 1,0 gl^{-1}, and babassu oil, 50,0 ml l^{-1} . For values (+1), the concentration were: yeast extract, 10,0 gl^{-1}, ammonium sulfate, 10,0 gl^{-1}, urea, 5,0 gl^{-1}, potassium phosphate, 10,0 gl^{-1}, and babassu oil, 75,0 ml l^{-1} . The compounds concentrations values for the central point were: yeast extract, ammonium sulfate, and potassium phosphate, 5,5 gl^{-1} , and urea, 3,75 gl^{-1}, (wt/vol); and babassu oil, 62,5 ml l^{-1} , (vol/vol). All media were adjusted to a pH 5.3 prior to sterilization by heating for 15 min. at 121^0 C. All fermentations were conducted in Erlenmeyer flasks with a capacity of 1000 ml containing 300 ml l^{-1} of growth media. Immediately after the inoculation of 5% of 10^7 cells/ml, the flasks were incubated for 96 hours at 28°C in an orbital shaker at 150 rpm. The growth profile was accompanied by biomass and pH determination, and emulsification activity.

2.3 Assay of emulsification activity

Emulsification activity was determined using the method developed by Cirigliano & Carman [4]. An aliquot of cultures was filter sterilized using a Millipore 0.22μm membrane filter. This sample (2 ml) was diluted with 2 ml of 0.1 M sodium acetate buffer (pH 3.0) and 1 ml of n-hexadecane was added. The mixture was shaken in a vortex for 2 minutes and allowed to stand for 10 min. The turbidity (measure of oil dispersion) was measured spectrophotometrically at 540 nm. The absorbance was multiplied by the dilution and expressed as emulsification activity.

2.4 Determination of the dry weight of the culture

Culture samples (10 ml) were filtered through previously weighed nitrocellulose filters (0.22 μm-pore-size). After removal of the medium, the filters were washed with demineralized water, dried in an oven at 70°C for 24 h, cooled in a desiccator and weighed. They were then repeatedly re-weighed until a constant dry weight was obtained. Duplicate determinations gave results that varied by < 1%.

2.5 pH measurement

Samples were taken from the cultures when fermentation had ceased. When the samples had been brought to a uniform temperature of 25°C, the pH of the supernatants was measured using an Orion, model 310, pH meter.

2.6 Factorial design

Three factorial two-level designs were used in the course of these assays. A replicated central point was added to each design, to allow for statistical significance calculations. Factorial design 1- a $2^{(5-1)}$ fractional factorial design based on five experimental factors at two levels, was used to evaluate culture conditions for biosurfactant production (the variable response). Factorial design 2 - a full 2^4 factorial design based on four of the five variables was used for a follow-up study. Finally, a full 2^3 factorial was performed to assess the effect of a lack of urea on the culture medium. For responses, the emulsification activity, were detected in the supernatants for every fermentation run was used. The effects were calculated from optical density values (OD-A540).

2.7 Statistical analysis

All data were analyzed using the Statistica software package [5]. The statistical significance of the results was established at the $p<0.05$ levels.

3. Results and discussion

3.1 Factorial designs and growth

An experimental design was used to determine the best concentrations of the media components that have previously been used. This study was accomplished in three consecutive steps: first, a fractional factorial design in a set of 16 experiments with two center points, in duplicate, was performed to determine the variables that could affect biosurfactant production. Then a 2^4 design was performed to evaluate medium performance in the absence of the nitrogen source, ammonium sulfate, which showed a negative effect on production, in the initial analysis. Finally, a 2^3 design was performed under the same conditions, but with the further exclusion of urea, to evaluate the effect of the remaining three factors on biosurfactant production. Despite the somewhat large variability of the replicate responses, several main and interaction effects we found to be significant at the 95% confidence level. In the first (2^{5-1}) design, the best responses for biosurfactant production were obtained at runs 8, 10, 13, 14, with 5.060, 4.950, 4.932 and 4.844 of absorbance at 540nm, respectively [Figure 1a]. The contrasts associated with the main effects of the five factors (yeast extract, urea, sulfate, phosphate and babassu oil) were calculated, and the main effects of phosphate, yeast extract, urea and babassu oil were all found to be positive, while that of ammonium sulfate proved negative, as shown in the Pareto plot in [Figure 1b]. The interaction of the factors ammonium and babassu oil show significant effect negative, indicating that the response decrease when these variables were used in a +1 level. On the other hand, the interactions between yeast extract and urea, show significant effect positive, indicating an increase over the emulsification activity when these factors were used in a +1 level. Thus, an interesting conclusion of the first part of this study was that for the best conditions for biosurfactant production yeast extract, phosphate, and urea should be used at +1 levels and ammonium at the -1 level [Figure 1b]. The purpose of this diagnostic two-level design was to obtain experimental data which served as an initial approach to the maximal biosurfactant production, establishing which factors had significant effects on response (biosurfactant production evaluated by the measurement of emulsification activity in the supernatant of culture) and whether these effects were positive or negative.

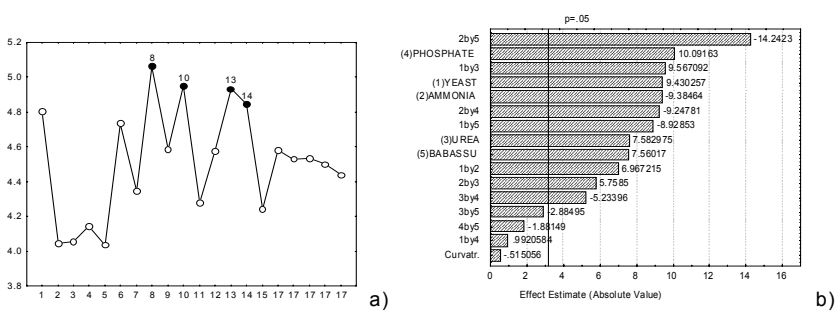

Figure 1a. Responses obtained for the $2^{(5-1)}$ fractional factorial design of biosurfactant production by *Candida lipolytica* UCP 0065 estimated as OD (A540), as a function of emulsification activity of the culture supernatant in each run.

Symbols: ○ shows the result obtained in each run, estimated as OD (A540); ● the high emulsification activity, estimated as OD (A540).

Figure 1b. Bar graph of standardized effects of the different variables tested in the experiment on biosurfactant production by *Candida lipolytica* UCP 0065.

The variables tested were the culture the culture medium compounds, yeast extract (1), ammonium sulfate (2), urea (3), potassium phosphate (4), and babassu oil (5). The point at which the effect estimates were statistically significant (at P= 0.05) is indicated by the vertical dotted line.

The values indicate the significant effects (positive or negative) of the factors and interactions between the variables.

The most significant effects were found with the water-insoluble carbon source, and the phosphate, linked by the energy production routes of the cell metabolism in the *Candida bombicola* growth [6]. Indeed, the carbon source is very important in the biosurfactant production process, especially the immiscible ones [7]. The importance of phosphate in the same process is also related by Reisfeld et al. [8], suggesting that more rapid dispersion of oil in seawater took place in buffered sea water, catalyzed by phosphate.

A 2^4 factorial design, excluding ammonium sulfate, that shows a negative effect in the first design; was subsequently carried out to determine the influence of the other four factors: yeast extract, urea, potassium phosphate and babassu oil, at the same levels. The responses obtained using this design are plotted in Figure 3-A. Babassu oil and yeast extract were found to have significant ($P<0.05$) effects on the response. The best conditions for biosurfactant production were those of run 12. Among the four variables investigated, the factors babassu oil and yeast extract had the most important effect on biosurfactant production (Figure 3-B). Babassu

oil has previously furnished good results in this process, and yeast extract was found to be essential for cell growth. The analysis shows that significant interactions between factors occurred for babassu oil x yeast extract, yeast extract x phosphate and yeast extract x urea, basically replicating the conclusions reached using the first design.

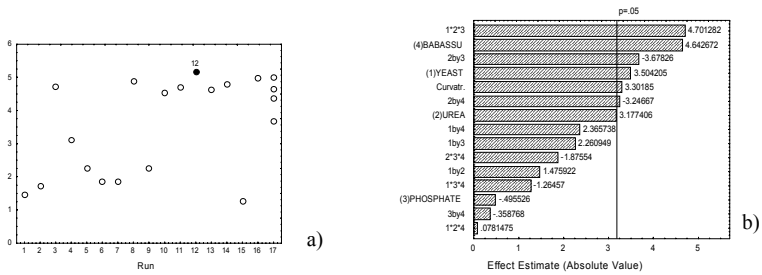

Figure 2a. Responses obtained for the full factorial design 2^4 for biosurfactant production by *Candida lipolytica* UCP 0065 estimated as OD (A540), as a function of emulsification activity of the culture supernatant in each run.
Symbol: ○ shows the result obtained in each run, estimated as OD (A540).
Symbol: ● shows the run result that obtained the high emulsification activity, estimated as OD (A540).
Figura 2b. Bar graph of standardized effects of the different variables tested in the experiment on biosurfactant production by *Candida lipolytica* UCP 0065.
The variables tested were the culture the culture medium compounds, yeast extract (1), urea (2), potassium phosphate (3), and babassu oil (4).
The point at which the effect estimates were statistically significant (at P= 0.05) is indicated by the vertical dotted line.
The values indicate the significant effects (positive or negative) of the factors and interactions between the variables.

In the final 2^3 design, the most significant biosurfactant production occurred in run 8, as indicated by the high absorbance, the parameter used to detect the emulsification activity (AE) (Figure 3a). The significance of the carbon source is shown in Figure 3b. The only nitrogen source in this experiment is yeast extract, which contains organic growth nutrients to supply some vitamins, [9], and mineral salts contained in seawater, an ingredient of the liquid phase medium. The economics of the production of biosurfactants must be worked out, if they are to compete with their chemical counterparts. The choice of inexpensive and renewable raw materials is important for the overall economic viability of the process, because these account for fifty percent of the final production cost [10].

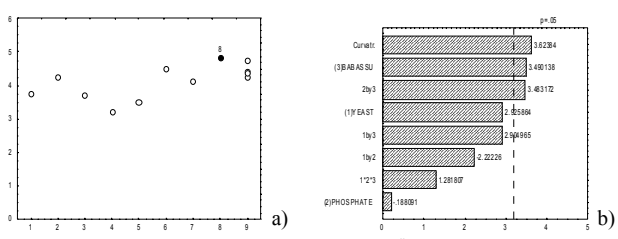

Figure 3a. Responses obtained or the full factorial design 2^3 for biosurfactant production by *Candida lipolytica* UCP 0065 estimated as OD (A540), as a function of emulsification activity of the culture supernatant in each run.
Symbols: ○ shows the result obtained in each run, estimated as OD (A540); ● the high emulsification activity, estimated as OD (A540)
Figure 3b. Bar graph of standardized effects of the different variables tested in the experiment on biosurfactant production by *Candida lipolytica* UCP 0065.
The variables tested were the culture the culture medium compounds, yeast extract (1), potassium phosphate (2), and babassu oil (3).
The point at which the effect estimates were statistically significant (at P= 0.05) is indicated by the vertical dotted line.
The values indicate the significant effects (positive or negative) of the factors and interactions between the variables

Babassu oil is of edible standard, does not contain preservative oils, and is used for cooking in the North region of Brazil. The relationship between metabolite production and the growth of the strain producer has been observed by Leal-Sánchez et al. [11]. They have shown that there is no clear-cut correlation between maximal Bacteriocin production and the numbers of *Lactobacillus plantarum* LPCO10 cells at the same points. These results are similar to ours. This may have been because cells for biosurfactant biosynthesis rather than for the growth consumed the nutrients. As expected, the capacity to sustain the large fluxes of carbon and energy required for rapid metabolite production appears to be inversely related to the growth efficiency of

microorganisms, principally in the case of metabolites like biosurfactants, which are composed of moieties with significantly different oxidation states [12]. This observation has been previously related by Rosenberg et al. [13], whose fermentation studies using oils as a carbon source demonstrated that growth rate can be limited by the interfacial surface area between water and oil. Other studies confirm that biosurfactant production is related to slowly growing cells [14], [15]. With respect to biosurfactant production by other microrganisms, we do not found results obtained in the similar conditions, for comparison to ours in the literature.

4. Conclusions

These results show that all the factors studied (yeast extract, urea, ammonium sulfate, potassium phosphate and babassu oil) appear to influence biosurfactant production, and higher biosurfactant synthesis is obtained when yeast extract, urea, potassium phosphate are at higher levels, while ammonium sulfate is at a lower level. For babassu oil, the level selected is of no consequence for the range studied here. This indicates that the combined effect of all the independent variables significantly contributes to the enhancement of biosurfactant production. Based on the experimental results it can be concluded that *Candida lipolytica* is able to use babassu oil as an insoluble carbon source as a substrate for producing biosurfactant. Therefore, it is feasible to use relatively inexpensive and renewable substrates for biosurfactant production.

Acknowledgements- The authors are grateful to the Brazilian agencies CNPq, CNPq/CTPETRO, FINEP,UNICAP and LANAGRO-PE for financial support.

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Surface active agent produced by *Candida lipolytica* using cassava flour wastewater as substrate

R. F. Silva Andrade[1,2], J. M. Luna[2,4], R. D. Rufino[2,3], C. D. Costa Albuquerque[2] , L. A. Sarubbo[2] and G. M. de Campos-Takaki[2]

[1] Biological Sciences Post-graduation Course, Catholic University of Pernambuco, Brazil
[2] Nucleus of Research in Environmental Sciences (NPCIAMB), Catholic University of Pernambuco, Recife-PE, Brazil
[3] Fungal Biology Post-graduation Courses, Federal University of Pernambuco, Recife-PE, Brazil
[4] Biological Sciences, Post-graduation Courses, Federal University of Pernambuco, Recife-PE, Brazil

The production of a biosurfactant by *Candida lipolytica* was carried out using cassava flour wastewater as a low-cost substrate. A factorial design was previously carried out to investigate the effects and interactions of the concentrations of cassava flour, ammonium sulphate and urea on the surface tension after 96 h cultivation. Maximum surface tension reduction (26.35 mN/m) was achieved for the medium supplemented with 10% cassava flour, 0.2% ammonium sulphate and 0.1% urea. The biosurfactant containing cell-free broth retained its emulsification properties after incubation at a wide temperature range and under specific concentrations of NaCl and pH values. The isolated biosurfactant seemed to be a carbohydrate-protein-lipid complex. The cell-free broth containing the biosurfactant removed 62.2% of motor oil adsorbed in sand, while the whole metabolic broth removed 81.7% of the oil, indicating that cassava flour wastewater is a rich and economic substrate for producing an effective biosurfactant applicable to bioremediation processes.

Keywords Biosurfactant; *Candida lipolytica*; Cassava flour wastewater

1. Introduction

Surfactants, amphiphylic molecules consisting of a hydrophylic head and a hydrophobic tail, are the active ingredients found in soaps and detergents. Due to their ability to concentrate at the air-water interface, they are commonly used to separate oily materials from a given medium. Surfactants increase the aqueous solubility of hydrophylic molecules by reducing their surface/interfacial tension at air-water and water-oil interfaces [1].

The commercial importance of surfactants is evidenced by the upward trends in producing them and the increasing number of industrial applications which use them. Almost all surfactants being currently produced are chemically derived from petroleum. However, these synthetic surfactants are usually toxic themselves and are difficult to degrade using microorganisms [2].

Even though interest in biosurfactants is steadily increasing, these compounds still do not compete economically with synthetic surfactants. To reduce production costs, different routes could be investigated such as by increasing yields and product accumulation; developing economic engineering processes, and using cost free or cost-credit feedstock to grow microrganisms and produce surfactants. This paper describes the application of a factorial design for enhancing the production of biosurfactant by *Candida lipolytica* UCP 0988, using cassava flour wastewater as a low-cost substrate.

2. Materials and Methods

Candida lipolytica UCP 0988 was kindly supplied from the Culture Collection of the Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil.

The micro organism was maintained in an anamorph state at 5°C on Yeast Mold Agar (YMA) slants containing (w/v): yeast extract -0.3%, malt extract - 0.3%, peptone - 0.5%, glucose - 1% and agar - 2%. Transfers were made to fresh agar slants each month to maintain viability. The *Candida lipolytica* was grown on solid medium at 27°C for 72 h; then, a loopful of the cream-colored culture was transferred to Erlenmeyer flasks of 250-ml containing 50-ml of the liquid medium Yeast Mold Broth (YMB) and incubated aerobically for one day at 27°C on a rotary shaker (150 rpm). The YMB culture contained 10^7 cells/ml and was used to initiate growth in the liquid Yeast Water Medium-YWM, composed of ammonium sulphate and urea dissolved in distilled water supplemented with cassava flour wastewater.

The biosurfactant production was evaluated using a statistical design. A 2^3 full factorial design was carried out to verify the effects and interactions of cassava flour concentration, ammonium sulphate concentration and urea concentration on the production of biosurfactant. In this design, a set of 12 experiments, with four

replicates at the central points, was performed. The range and levels of the components (factors or independent variables) under study are given in Table 1. The production experiments were carried out using Erlenmeyer flasks (500 ml) containing 100 ml of culture medium incubated for 96 h under orbital shaker (150 rpm). After this period, aliquots were used to measure surface tension, emulsification index and emulsification activity (U.A.E.) on the cell-free broth obtained centrifuging the cultures at 5000 rpm for 20 minutes. The surface tension was determined with a Tensiometer model Sigma 70 (KSV Instruments LTD - Finland) using the Du Nouy ring method at room temperature. The emulsification index was analyzed according to Cooper and Goldenberg [3], whereby 2 ml of cotton oil was added to 2 ml of the cell-free broth in a graduated screw cap test tube and vortexed at high speed for 2 min. Negative control of emulsification was carried out using water. Emulsion stability was determined after 24 h, and the emulsification index (E) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100. Emulsification activity was determined using the method described by Cirigliano and Carman [4]. The cell-free broth (2ml) obtained after centrifugation was diluted with 2ml of 0.1M sodium acetate buffer (pH 3.0) and 1ml of one of the following substrate corn oil. The mixture was placed in a screw-capped tube and shaken for 2 min at 27 °C. The resulting uniform emulsion was allowed to sit for 10 min, after which its absorbance was measured at 540 nm. The blank used contained 2 ml of sterile production medium. One unit of emulsification activity was defined as the amount of emulsifier that affected an emulsion with an absorbance at 540 nm of 1.0. Stability studies were conducted after selection of the best condition for biosurfactant production according to the factorial design using the cell-free broth after 96 hours cultivation. Samples were maintained at 0 ºC, 5 ºC, 70 ºC, 100 ºC and 120 ºC for 10 minutes and cooled to room temperature, after which the emulsification index was measured. To study the pH stability, the pH of the cell-free broth was adjusted to different pH values (2 to 12) and the emulsification index was measured. The effect of NaCl concentration (2 to 12%) on the emulsification index of the cell-free culture broth was also determined. For quantitative determinations, the biosurfactant was isolated by acid precipitation with chloridric acid according to Nitschke and Pastore [5]. Subsequently, the sample was centrifuged at 5.000 rpm for 20 min at 28 °C and liophylized. Protein in the isolated biosurfactant was estimated by using the total protein test kit from Labtest Diagnóstica S.A., Brazil. Sugars were measured by the phenol l-sulphuric acid method using glucose as standard [6]. The lipid composition of the crude bioemulsifier was determined according to Manocha [7]. The removal of motor oil (10 ml) adsorbed in a sand sample (20.0 g) was conducted with 300 ml the cell-free broth and the whole broth obtained after 96 hours, during 48 hours at 150 rpm, according to Nitschke and Pastore [5].

3. Results and Discussion

3.1 Production of the biosurfactant

The production of the biosurfactant was conducted using a factorial design, as described in Table 1. The numbers from one (1) to eight (8) correspond to the experimental conditions obtained from the combination of the variables tested and the numbers nine (9) to twelve (12) correspond to the conditions of the central point. From all the runs carried through, the run number two had shown the higher surface tension reduction, while the run number five showed the higher emulsification index. Among these two runs, the one represented by the number two (10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea) was selected for further studies of production and characterization of the biosurfactant, once it conducted to a better surface tension value and also great emulsification index and activity values, which indicate that the surface agent produced has attractive properties as ability to reduce substantially the surface tension and to act as an emulsifier.

Table 1 Surface tension, emulsification index and emulsification activity values obtained in the 2^3 full factorial design used for biosurfactant production by *C. lipolytica* grown in Yeast Water Medium during 96 hours. Numbers one (1) to eight (8) are the runs corresponding to the experimental conditions obtained from the combination of the variables tested and numbers nine (9) to ten (12) are the runs corresponding to the experimental conditions of the central point

Conditions	Cassava flour wastewater (%)	Ammonium sulphate (%)	Urea (%)	Emulsification index of cotton oil (%)	Emulsification activity of corn oil (U.A.E)	Surface tension (mN/m)
1	5.0	0.2	0,1	30.35	2.024	48.33
2	10.0	0.2	0.1	26.5	1.934	26.35
3	5.0	1.0	0.1	21.42	2.048	46.80
4	10.0	1.0	0.1	16.66	2.048	37.61
5	5.0	0.2	0.3	28.57	2.055	46.19
6	10.0	0.2	0.3	18.75	2.025	46.49
7	5.0	1.0	0.3	20.28	1.705	43.98
8	10.0	1.0	0.3	28.57	2.007	42.59
9	7.5	0.6	0.5	25.00	2.040	46.97
10	7.5	0.6	0.5	25.00	2.038	46.91
11	7.5	0.6	0.5	30.36	2.015	47.02
12	7.5	0.6	0.5	33.33	2.023	47.12

3.2 Effect of variables used on the surface tension

Figure 1 illustrates the Pareto Chart, with 95 % confidence level, for effect estimates, in absolute values. From the chart, it can be seen that the cassava flour wastewater influenced positively the reduction of the surface tension in the range studied, while urea and ammonium sulphate did not significantly influence the surface tension reduction. The association between the variables cassava flour wastewater and urea has been statistically significant, acting negatively in reducing the surface tension after 96 hours of cultivation.

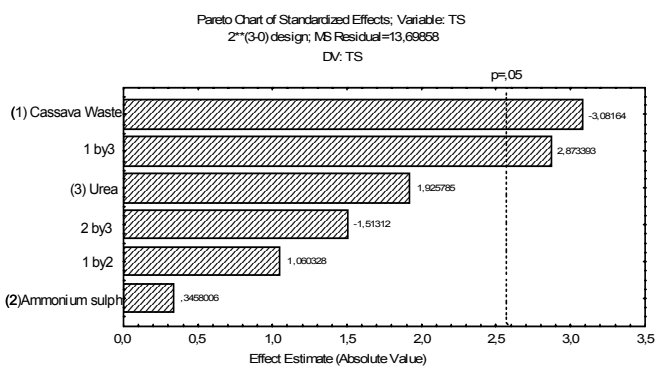

Fig. 1 Pareto Chart of standardized effects for surface tension of the cell-free broth from *C. lipolytica* after 96 hours of cultivation for the 2^3 full factorial design. The point at which the effect estimates were statistically significant (at p = 0.05) is indicated by the broken vertical line

3.3 Stability of biosurfactant according to the emulsification index

The stability tests were conducted for the biosurfactant produced in the medium formulated with 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea, according to the previous factorial design. The tests conducted on the cell-free broth, regarding the variation of temperature, demonstrated an effective stability of the emulsification indexes in the temperature range studied in this work (Figure 2) although an increase of 15% of activity had been observed at 120 ° C (Figure 2). Similar behavior towards stability was also observed for the biosurfactants produced by *Bacillus subtilis* [8] and *Nocardia* SP. L-417 [9] when the cell-free broths were heated to 100 °C. Liposan of *Candida lipolytica* was found to be relatively stable between 30 and 90 ° C, but lost 60% of its activity after being boiled for 1 hour [4] Regarding the influence of pH on the emulsification

index of the cell-free broth containing the biosurfactant (Figure 3), there was a wide variation in all tracks tested with stability only in pH 6. The addition of NaCl did not show a stability pattern of the emulsification, once the percentile values remained unaltered in the presence of 2, 6 and 10 % of salt in the cell-free broth containing the biosurfactant (Figure 4). The biosurfactant from *C. glabrata* also achieved stability in the presence of 10% of NaCl [10].

3.4 Chemical composition of biosurfactant selected

The biochemical analysis of the biosurfactant produced by *C. lipolytica* in the medium formulated with 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea showed the presence of carbohydrates and proteins, and lipids in a minor quantity. Others emulsifiers produced recently by *C. lipolytica* seem to be also polysaccharide-protein-lipid complexes [1, 10].

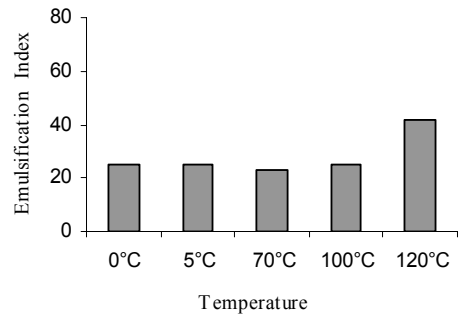

Fig. 2 Effect of temperature on the emulsification index (%) of corn oil by the cell-free broth of *C. lipolytica* grown in medium containing 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea during 96 h

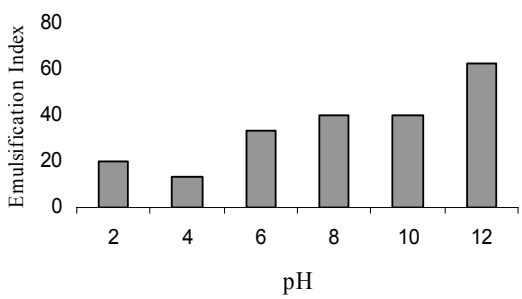

Fig. 3 Effect of pH on the emulsification index (%) of corn oil by the cell-free broth of *C. lipolytica* grown in medium containing 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea during 96 h

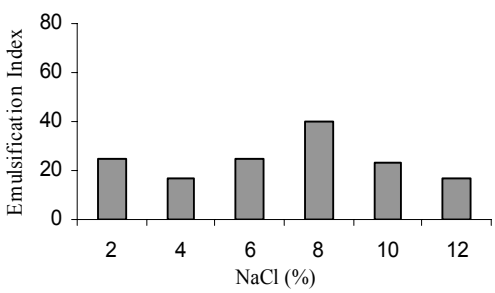

Fig. 4 Effect of NaCl addition on the emulsification index (%) of corn oil by the cell-free broth of *C. lipolytica* grown in medium containing 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea during 96 h

3.5 Application of the biosurfactant in the oil removal

The removal of motor oil from sand utilizing the cell-free broth containing the biosurfactant produced by *C. lipolytica* in the medium formulated with 10% cassava flour wastewater, 0.2% ammonium sulphate and 0.1% urea showed values of 62.2% while the whole broth removed 81.7% of the oil. Abu-Ruwaida et al. [11] showed that the cell-free broth containing a biosurfactant produced by *Rhodococcus* cells was able to recover 86% of crude residual oil adsorbed in the sand while Cameotra and Makkar [8] demonstrated that the biosurfactant isolated form *Pseudomonas aeruginosa* was able to recover 56% of the oil adsorbed in a packed column containing sand.

4. Conclusions

The results obtained from this study show that the biosurfactant produced by *C. lipolytica* cultivated in a low-cost medium has attractive properties such as low surface tension and emulsification capacity over a wide range of temperature and under specific NaCl and pH conditions. Its ability to recover oil from oil-saturated sand was also demonstrated. Thus, these characteristics indicate the potential for using the biosurfactant in the oil industry, especially in MEOR (Microbial Enhanced Oil Recovery). Studies are in progress to scale up growth conditions and biosurfactant production in a bioreactor.

Acknowledgements This work was financed by Funding Agency for Studies and Projects (FINEP), the National Council for Scientific and Technological Development (CNPq), and Catholic University of Pernambuco, Brazil.

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Biotechnologically Relevant Enzymes and Proteins

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Expression of different recombinant forms of the precursor of human pulmonary surfactant protein B (pro SP-B) in *Pichia pastoris*

F.Gil[1], E.J. Cabré[1], C. Pastor[2], P. Estrada[1], J. Perez-Gil[1]

[1] Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain

[2] Departamento de Alergias, Fundación Jimenéz Díaz, Madrid, Spain

Surfactant protein B (SP-B) is an essential constituent of pulmonary surfactant. Mature SP-B is a 79 amino acid hydrophobic peptide, associated with surfactant phospholipids in the alveolar airspaces, and it is absolutely required for life (1). Presence of SP-B permits a rapid and efficient formation of surface active films at the respiratory air-liquid interface of lungs, reducing the energy required for breathing. Lack of an operative surfactant results in several important pathologies, which are in some cases treated by supplementation with SP-B containing exogenous surfactant preparations. However, and despite of its importance in therapy, very little is known today about the basic requirements to express this protein in heterologous systems. The present work explores different strategies to produce proSP-B, the precursor of human SP-B, and some truncated variants, in the yeast *Pichia pastoris.*

Introduction

Pulmonary surfactant is a complex mixture of lipids and aprox. 10% (w/w) proteins produced and secreted by type II pneumocytes into the alveolar spaces. Protein fraction in surfactant consists of four specific polypeptides, two hydrophilic macromolecular assemblies, the collectins SP-A and SP-D, and two small very hydrophobic proteins, SP-B and SP-C. Once surfactant is secreted into the thin aqueous layer that covers the alveolar epithelium, it plays an essential role in reducing surface tension at the air-liquid interface to prevent alveolar collapse at the end of expiration.

Human SP-B is synthesized by the pneumocytes as a preproprotein (prepro SP-B) of 381 amino acids, including a N-terminally cleavable signal peptide of 23 residues, which allows translocation into the endoplasmic reticulum (ER). Along the exocytic pathway of the type II cells, proSP-B (residues 24-381) is proteolytically cleaved in different steps [2,3]. Cleavage of N-terminal (24-200) and C-terminal (280-381) flanking domains generates the biophysically active mature SP-B. Surfactant protein B (SP-B) is an 8-kDa hydrophobic protein, positively charged, involved in formation and stability of the surface active phospholipid film which prevents collapse of lung alveoli during respiration. Presence of SP-B is essential for the surface tension lowering properties of pulmonary surfactant and for normal respiratory function. Absence or inactivation of SP-B in animal models results in respiratory distress leading ultimately to respiratory failure, and inherited deficiency of SP-B is a lethal disorder in neonates.

Thus, proSP-B and its different forms are transient intermediates in SP-B synthesis and do not accumulate in type II pneumocytes. Isolation and purification of this protein from natural sources in sufficient amounts to approach its structural and functional characterization is therefore not feasible, making necessary the development of strategies to express mature SP-B or some of its intermediates as recombinant proteins in heterologous systems. Our goal in the present study was the expression of proSP-B and a set of different truncated forms, mimicking the natural intermediates generated across the exocitic pathway, in the methyllotrophic yeast *Pichia pastoris*. The analysis of both mRNA and protein expression of these different recombinant forms open new perspectives in the design and development of future surfactant-based therapeutical applications.

Material and Methods

E. Coli strain DH5α was used as an intermediate host for the plasmid constructions. pKC4 vector kindly provided by Dr. T. Weaver (University of Cincinnati, USA), containing the 2.0 kb full-length human proSP-B cDNA, was used as a PCR template for obtaining the different DNA fragments. Segments selected included the complete proSP-B sequence (residues 24-381), the proSP-B sequence without the carboxyl terminal flanking domain (**Nt-Mat**, residues 24-280), the mature domain alone (**Mat**, residues 200-280), and proSP-B without the N.terminal flanking domain (**Mat-Ct,** residues 200-381) (Fig. 1). To amplify these DNA sequences, a new set of primers was designed (Fig. 1). After the first PCR, the amplified product corresponding to the different constructions was re-amplified with the specific primer that allows fusion of the protein encoding region in

frame with the secretion sequence α-mating factor (α-Factor), present in plasmid pPICZα, including also a Xho I restriction site. The reverse primer allows fusion with c-myc epitope and a His-tag extension, to facilitate the purification of the recombinant proteins, and contains an Xba I restriction site (Fig 1).

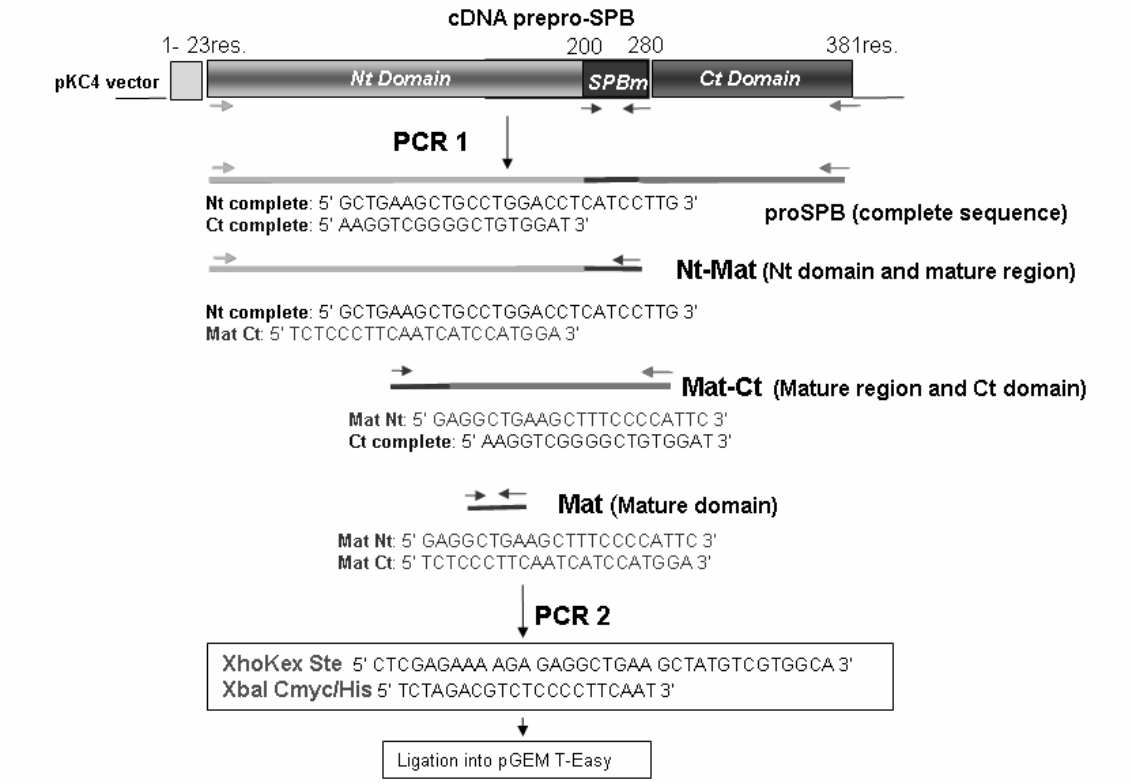

Figure 1. Human preproSP-B cDNA sequence. Schematic representation of the different DNA fragments amplified by PCR and specific primers from the complete sequenced of cDNA encoding for human SP-B precursor

The amplified DNA fragments were purified, ligated into pGEM-Teasy vector (Promega) and used to transform DH5α *E. Coli* cells. Plasmid DNAs from several colonies were then sequenced. The intermediate vectors were digested with Xho I and Xba I restriction enzymes, subcloned in the same sites into pPICZα plasmid, and newly sequenced to confirm the in-frame arrangement of the leader sequence and proSP-B or its truncated forms, as well as the fusion with the c-myc epitope and the His tag extension. The *P. pastoris* expression vector pPICZα, has an expression cassette under the Alcohol oxidase promoter (AOX 1 Pr), which drives the expression of the all our constructions under methanol induction, (Fig. 2). The AOX pr fragment has 942 bp. and targets plasmid integration in the AOX locus. In addition, pPICZα contains Sh ble gene for zeocin resistance and selection.

For the preparation of competent *Pichia* X-33 cells, Pichia EasyComp Kit (Invitrogen) was used following manufacturer´s instructions. For the yeast transformation, 3 ug of all pPICZα/recombinants were linearized with specific restriction enzyme, and the purified larger fragment was used to transform X-33 cells by replacement-type DNA integration. Transformed cells were incubated on Yeast extract Peptone Dextrose medium (YPD) plates, containing 100ug/ml Zeocin for resistance selection, at 30ºC for 3-5 days until colonies appeared.

Yeast DNA was extracted from transformed cells, using the Easy DNA Kit for genomic DNA isolation (Invitrogen) and used as a PCR template. Samples of PCR product were loaded into 1% agarose gel for electrophoresis separation and separated bands were visualized using ultraviolet light after staining with ethidium bromide (1μg/ml) in tris-acetate-EDTA (TAE) buffer .

Expression experiments were conducted based on the procedure outlined in Invitrogen's Easy Select *Pichia* expression manual, with some modifications. The transformed cells were cultured overnight at 30ºC in 200 ml of buffered complex medium containing glycerol. Cells were then sedimented by centrifugation and later resuspended until an OD_{600} of 1.0 in buffered complex medium containing methanol to induce the AOX promoter. This culture medium was maintained for 4 days and supplemented daily with 5ml of methanol per litre of culture. Aliquots of expression medium were removed at different times. The cultured medium of X-33-induced cells was cleared of yeast cells by centrifugation at 3000 g for 10min. at 4ºC. Samples of proteins

secreted into the supernatant of the culture medium were precipitated with acetone and loaded on SDS/PAGE. In addition, yeast cells were processed using a Yeast Cell Lysis/Extraction Kit (CelLytic-Y SIGMA) and analysed by SDS/PAGE and Western blotting.

SDS-PAGE analysis was performed using 12-14% gels [4], using a Mini Protean II System (Bio-Rad). For Western blotting, bands in the gel were transferred to a Hybond ECL Nitrocelulose Membrane (Amersham Biosciences) using a Trans-Blot Sd semi-dry electroblotting apparatus (Bio-Rad) set at 20 V for 20 min in 25 mM tris, 192 mM Glycine and 20% of methanol. Transblotted membranes were probed with mouse monoclonal anty-polyhistidine peroxidase conjugate clone His-1 (SIGMA), which recognizes the his-tag extension of the recombinant proteins. In parallel with the use of anti-His antibody, transblotted membranes were also probed with specific antibodies (a gift from Prof. Weaver, Cincinnati University) developed against epitopes on N-terminal or C-terminal flanking domains of proSP-B. Besides, other specific commercial anti-SP-B antibody (Chemicon) was employed.

In order to facilitate the release of RNA from the transformed yeasts, the cells were subjected to mechanical disruption using a high-speed bead beater; RNA was then extracted from disrupted cells according to the protocol of the Qiagen RNeasy extraction kit (Qiagen). All samples were subjected to on-column DNase digestion using a RNase-free DNase treatment (Qiagen) during RNA isolation. RNA samples were analyzed in 1,4% agarose-formaldehyde gels for RNA electrophoretic separation and visualized using ultraviolet light after staining in ethidium bromide (1μg/ml) in MOPS 1X buffer. Isolated RNA was used as a template in reverse transcriptase (RT-PCR) analysis, conducted in two steps. First, the synthesis of the cDNA strand was performed using the SuperScript II RT kit (Invitrogen). Briefly, 1μg of RNA samples and oligo (dT) primers were employed. After adding the reverse transcriptase enzyme, the reaction was carried out at 42ºC for 1 hour and terminated after 10 min at 65ºC. The cDNA produced from the RT reaction was then used as a template for amplification by PCR using specific primers for each construction, as previously described. Samples were then analyzed in 1% agarose gel for electrophoresis separation.

Results

The DNA fragment encoding proSP-B (residues 24-381) and the rest of the constructions encoding different combinations of the mature SP-B module and some of the flanking propeptides, were inserted into the yeast expression vector pPICZα as described in Material and Methods. The recombinant vectors were then sequenced, confirming the in-frame arrangement of the leader sequence (α-factor) and the sequence of proSP-B or its derivates, as well as the fusion with the c-myc epitope and the His tag extension (Fig. 2A).

All recombinant vectors were used to transform X-33 cells by replacement-type DNA integration. Transformed cells were incubated on YPD plates, containing 100 ug/ml Zeocin for resistance selection, at 30ºC for 3-5 days until colonies appeared (Fig. 2A).

Genomic DNA from transformed yeast was isolated and PCR analysis was performed for every construction. Agarose gel electrophoresis of the PCR products showed in most of the transformed yeast that pPICZα expression cassettes containing the constructions were integrated into the *P. pastoris* host cell genome (Fig. 2B). To test the ability to produce recombinant proteins, three or four PCR positive clones of each construction were selected and analysed for the production of recombinant proteins. Expression experiments were conducted based on the procedure outlined in Invitrogen's Easy Select *Pichia* expression manual. Different samples were taken from cultures transformed with all the constructions at different times post-induction and were tested for the presence of recombinant proteins, both in the culture medium and in the cells. Secreted proteins were analyzed with several antibodies, but no reactive bands corresponding to the recombinant proteins were detected in the culture medium (data not shown).

In order to analyze whether the transformed cells were really expressing the different recombinant proteins, yeast cells were processed and total cellular proteins were tested by Western Blot analysis Representative blots analyzing intracellular expression of the different constructions in transformed *P. pastoris* are shown in Fig. 3.

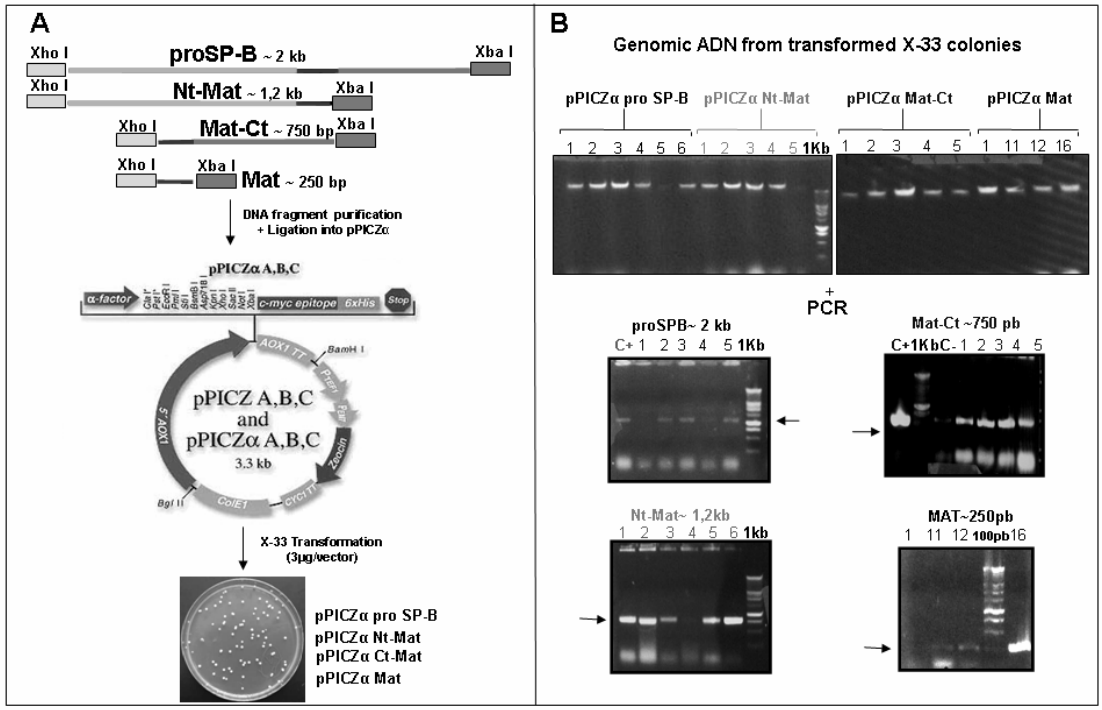

Figure 2.A Obtention of recombinants pPICzα vectors. DNA fragments obtained after enzimatic digestion (XhoI/XbaI) of pGEM recombinant vectors containing each of the proSP-B constructs were inserted into yeast expression plasmid pPICzα. The scheme shows the additional elements fused to the different proSP-B constructs. A picture illustrating colonies of X-33 P. pastoris transformed with pPICzα proSP-B is also included. **2.B** Integration analysis of proSP-B in X-33cultured cells. Isolated genomic DNA of transformed X-33cells, potentially bearing the different proSP-B constructions were subjected PCR analysis with proper primers. Arrows indicate the expected position of the PCR for each constructions and numbers in the gel lanes refer to the different colonies checked.

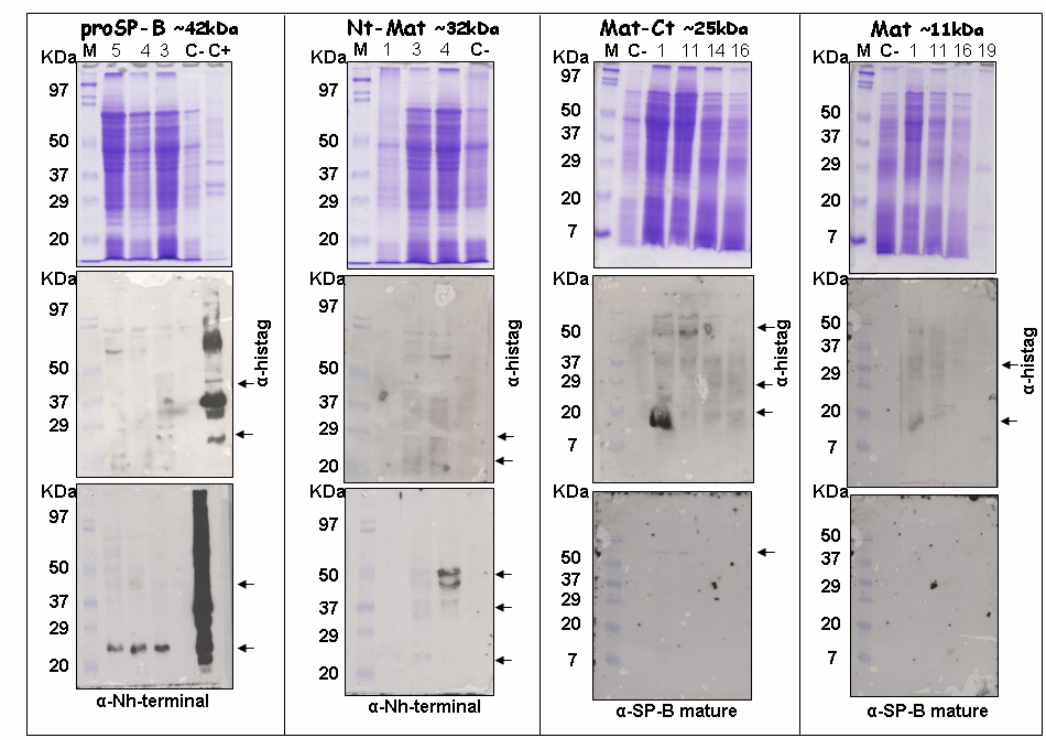

Figure 3. Analysis of the protein expression in transformed X-33 cells. Panels above show Coomassie Blue stained SDS-PAGE gels of selected X-33 colonies transformed with the different proSP-B constructs. Lower panel pictures show illustrative Western Blots of the colonies, develope with primary antibodies against His-tag extension, the N†- flanking domain of proSP-B or the mature SP-B protein.

Yeast X-33 cells transformed with the complete proSP-B presented some reactive bands, most of them not corresponding with the expected molecular weight of the recombinant proSP-B polypeptide (~42 kDa). An intense band of around 24 kDa. was detected with anti-Nt antibody. Similar results were found with Nt-Mat recombinant protein (~32 kDa). On the other hand, the cells from the other two constructions presented some weak reactive bands corresponding to apparent similar size to that expected for the mature domain of SP-B (9-11 kDa) or the fusion of SP-B and its Ct flanking domain (~25 kDa). The lack of strong expression evidences, in these experiments, could be due to either lack of protein translation or lack of accumulation of the recombinant proteins.

In order to check whether the recombinant DNA sequences inserted into the yeast genome were actually being transcribed, reverse transcriptase analysis was performed with material from the different cells. The results of RT-PCR experiments detect the product of the transcription of the different DNA sequences inserted in genome of the transformed cells. Transcription of the different constructions were demonstrated by the presence in agarose gel of DNA bands of ~2 kb (proSP-B), ~1,2 kb (Nt-Mat), ~750 bp (Mat-Ct) and 250 bp (Mat) corresponding to the different recombinant DNA sequences (Fig. 4).

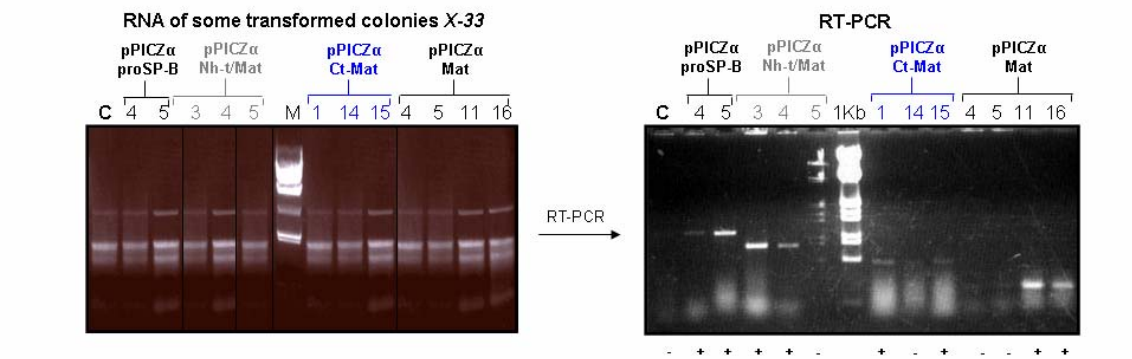

Figure 4. Detection of mRNA encoding proSP-B constructs in transformed X-33 cells by RT-PCR analysis. Left, gel was run with total RNA from selected colonies transformed with the different proSP-B containing vectors. The gel on the right side, analyses the products RT-PCR using total RNA from the colonies as template. ***C*** negative control (untransformed colony), ***M***, RNA molecular size makers, ***1Kb***, DNA size markers. The numbers in the lanes corresponding to the different colonies tested (-, negative and +, positive for RT-PCR)

Discussion

The present results demonstrate that different recombinant constructions have been successfully integrated into the X-33 yeast genome and that *P. pastoris* system allows for transcription of human pro-SP-B cDNA and several recombinant derivates.

Although strong reactive bands of recombinant protein accumulated in transformed cells have not been detected in Western blots, the result of the RT-PCR experiments demonstrate that mRNA is being transcribed and thus that the lack of protein is due to problems with translation and/or folding and accumulation.

Three main reasons are cited for the lack of expression of biologically active proteins in heterologous systems: i) the host cell lacking components required for the translation of the recombinant protein, ii) activated proteolysis of the protein as it emerges from translation, or iii) improper folding of the heterologous protein, which is then targeted for elimination [5] .

On the other hand, Surfactant protein SP-B has strong affinity to interact with and perturb the structure of lipid, membranes, which could interfere with the trafficking of the recombinant proteins through the yeast secretory pathway. Our RT-PCR analyses suggest that activated proteolysis and/or improper folding of the hydrophobic SP-B domains could be dominating factors leading to the absence of production/accumulation of these recombinant proteins.

No previous studies have been reported, to our knowledge, on the utilization of a *P. pastoris* system for the expression of proSP-B precursor, processing intermediates and the mature SP-B. Further experiments already in progress will help to determine whether Pichia can be or not a successful expression system for surfactant proteins. Strategies to test in orther to improve intracellular expression will include preparation of fusion constructs of proSP-B modules to soluble yeast proteins, and the use of yeast retention signals to direct protein expression towards defined membrane organelle.

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Partial purification of xylose reductase from *Candida guilliermondii* for the use of the conversion of xylose into xylitol

Ester Junko Tomotani[1,2], Michele Vitolo[1], Priscila Vaz de Arruda[2], Maria das Graças de Almeida Felipe[2]

[1] School of Pharmaceutical Sciences, University of São Paulo, 05508-900, São Paulo, Brazil
[2] School of Engineering, University of São Paulo,12602-810, Lorena, Brazil

Xylitol is used as a natural sweetener in food products as well as a source of energy in infusion therapy and in the prevention of otitis media, osteoporosis, lung infection and myoadenylate deaminase deficiency. Xylose may be reduced to xylitol by NADPH-linked xylose reductase (E.C.1.1.1.21) (XR), a beneficial economic alternative for the catalytic hydrogenation of pure xylose, obtained from lignocellulosic hydrolisates. The purpose of this work is to evaluate the bioconversion of xylose to xylitol using purified XR and glucose 6-phosphate dehydrogenase as an auxiliary enzyme in order to reduce the cofactor NADP to NADPH required for the XR activity. For such a purpose, cells were disrupted by sonication and the supernatant was diluted to 1:4, 1:5 and 1:10 (v/v) for a further ultracentrifugation process using Amicon Ultra-15 Centrifugal Filter devices. The bioconversion of xylose into xylitol using both XR and G6PDH enzymes was done in a batch assay at room temperature. The partial purification yields of XR attained for 1:4, 1:5 and 1:10 dilutions were respectively 45.6, 64.5 and 67.2%. The use of both XR and G6PDH for the xylitol production reached a yield of 35.5%.

Keywords xylose reductase; purification; xylitol

1. Introduction

Xylitol is commercially used as a natural sweetener in several food products as well as being a source of energy in infusion therapy and in the prevention of otitis media, osteoporosis, lung infection and myoadenylate deaminase deficiency [1,2]. Although xylitol is found naturally in fruits, its extraction is difficult and uneconomical [1]. It is currently produced by a catalytic dehydrogenation of xylose which is extracted from sugar cane bagasse, corncobs or rice husks [1] which is also an expensive process due to the number of purification stages required [3].

Xylose may also be reduced to xylitol through the NADPH-linked xylose reductase yeast (E.C.1.1.1.21) (XR), a 36 kDa protein which acts at the beginning of a pentose pathway [4-6]. The use of XR in the xylose-xylitol conversion constitutes a more economical alternative for the catalytic hydrogenation of pure xylose, which is, at present, obtained from lignocellulosic hydrolysate. This conversion procedure is advantageous due to selective reduction of xylose to xylitol, low energy requirement and low environmental impact [2].

Therefore, a biotechnological process could lower the production costs by the direct use of hydrolysate under milder conditions of pressure and temperature due to the specificity of the enzymes or microorganisms acting on the xylose-xylitol conversion [7, 8].

Xylose reductase yeast is a member of the aldo-keto reductase family of enzymes, which are widely distributed in organisms such as mammals, fish, insects, amphibian and reptiles [9]. Among the xylose-fermenting yeasts, *Candida guilliermondii* is reported to be one of the most efficient xylitol-producers [4]. Although xylose reductase has high application potential and is available in the purified form at lab scale [10], it is not present in the market. Thus it must be prepared by extracting it from *C.guilliermondii* grown either in synthetic or hydrolysate medium.

This work is to purify xylose reductase from *Candida guilliermondii* which was cultivated in a batch process previously established [2] to evaluate a further bioconversion of xylose to xylitol. In order to guarantee a supply of NADPH for XR activity, the glucose 6-phosphate dehydrogenase (G6PDH) was used as an auxiliary enzyme to reduce the cofactor NADP to NADPH. XR extract is often contaminated by xylitol dehydrogenase (EC 1.1.1.9)(XDH), a constitutive enzyme in *C.guilliermondii* which oxides xylitol to D-xylulose in presence of NADP [4]. By coupling the G6PDH and XR activities, the medium will remain depleted of NADP along the reaction; thus the XDH activity is minimized. This procedure is necessary because the β-nicotinamide dinucleotide phosphate reduced form (NADPH) is far more expensive than the oxidized counterpart (NADP). Moreover, the coupled system (G6PDH/XR) would be an alternative and a promising form for the xylose-xylitol bioconversion using a continuous reactor such as the membrane reactor.

2. Material and Methods

2.1 Microorganism and inoculum preparation

This procedure was done based on Rodrigues *et al*. [2]. *C. guilliermondii* FTI 20037 was inoculated in a medium formulated with D-xylose (30.0g.L^{-1}) and supplemented with $(NH_4)_2SO_4$ (2.0g.L^{-1}), $CaCl.2H_2O$ (0.1 g.L^{-1}), and rice bran extract (10.0% v/v). The cells were cultivated in 125 mL Erlenmeyer flasks containing 50 mL of this inoculum medium. It was incubated on a rotary shaker (model Co-Innova 4000, New Brunswick Scientific, Edison, NJ) under an agitation of 200 rpm at 30°C for 24h. Afterwards, the cells were collected by centrifugation (2000 x g, 15min), washed with sterile water, centrifuged, and re-suspended in sterile water. An adequate volume of this suspension was removed to attain the desired inoculum concentration ($0.3x10^8$ cells.mL^{-1}, approximately 1 g.L^{-1}).

2.2 Medium and fermentation conditions

A synthetic medium consisted of xylose (50 g.L^{-1}) as a carbon source which was supplemented with $(NH_4)_2SO_4$ (2.0 g.L^{-1}), $CaCl.2H_2O$ (0.1 g.L^{-1}) and with a rice-bran extract (20 g.L^{-1}). The pH was adjusted to 5.5 with NaOH 2.0 M to be used as the fermentation medium. The fermentation was performed in a batch process at 30 °C in 125mL Erlenmeyer flasks containing 50mL of this fermentation medium, using a rotary shaker (model Co-Innova 4000, New Brunswick Scientific, Edison, NJ) under an agitation of 200 rpm for 34 hours. The cells were harvested by centrifugation at 2700 x g for 15 min, and re-suspended in 0.1 mol. L^{-1} phosphate buffer (pH 7.2). The obtained and final suspension (15.0 g.L^{-1}) was stored in a freezer until further procedures.

2.3 Enzyme assays

The suspension of *C. guilliermondii* (0.2g/mL) was disrupted by 20-kHz sonication in 1-second pulses for a period of 45 min using a disrupter (VC-100; Sonics & Materials, Newton, CT, USA) followed by a centrifugation at 6700 x g for 10 min at 4 °C. The supernatant was then diluted to 1:4, 1:5 and 1:10 (v/v) for a further ultracentrifugation process using an Amicon-Ultra-15-50K-NMWL (Nominal Molecular Weight Limit) Centrifugal Filter device. Ten milliliters of a diluted sample were added into an Amicon-Ultra-15-Centrifugal device and centrifuged at 10000 x g for 30 min at 4 °C. Afterwards, the filtrate was collected for protein and xylose reductase activity determinations.

The XR, XDH and G6PDH activities were determined spectrophotometrically at 340 nm at room temperature [11, 12].

One XR, XDH or G6PDH unit (U) was defined as the amount of enzyme which catalyzes the reduction/oxidation of 1 µmol NADP/min or 1 µmol NADPH/min, respectively. Specific activities were expressed as $U.mg_{prot}^{-1}$.

2.4 Bioconversion of xylose into xylitol

For the purpose of bioconversion, kinetic characterization of both XR and G6PDH was considered. The kinetic parameters adopted for XR was the one determined previously by Sene *et al*. [11], whereas G6PDH parameters were determined as follows.

A standard assay for G6PDH was carried out in a 1.2 mL cuvet of a spectrophotometer (Beckman-Coltermodel DU 640) according to Das Neves *et al.* [12]. The NADP and G6P concentrations of the standard reaction test related to G6PDH were changed at the following intervals in order to determine the kinetic parameters (K_M and V_{max}) through the conventional Lineweaver-Burk plots: NADP (2, 4, 5, 6, 8, 10, 12, 15 and 18 µM) and G6P (6, 12, 15, 18, 24, 30 and 45 µM). The pH was 7.4 and the temperature was maintained at room temperature for all assays.

The bioconversion of xylose into xylitol using both enzymes, partially purified XR (1 mL) and G6PDH (25.2 U) (from baker's yeast, 232 units/mg protein, Sigma G-6378), was performed in a batch assay for 3 hours at room temperature and at an agitation of 100 rpm. The reaction medium (50 mL) consisted of xylose (0.5 M), glucose 6-phosphate (0.3 mM), NADP (1.25 mM), and a Tris-HCl buffer solution (50 mM) at pH of 7.4 as well as the addition of the enzymes. Aliquot samples were collected every 20 minutes for the measurement of total reducing sugars (xylose and glucose-6-phosphate). All of the chemicals were of analytical grade.

2.5 Analytical methods

Cell growth was monitored by measuring absorbance at 600 nm using a spectrophotometer (Beckman-DU 640B; Beckman Instruments, Allendale, NJ, USA). Cell concentration was calculated based on the relationship of optical density and cell dry weight through a calibration curve [6]. Protein was determined based on the difference between UV absorbance measured at 215nm and 225nm, using bovine serum albumin (BSA, from Sigma) as a standard protein [13]. The consumption of the total reducing sugars was measured as described previously by Arruda and Vitolo [14].

3. Result and Discussion

3.1 File formats and templates

The obtainment of xylose reductase is an important step in the development of the enzymatic process for bioconversion of xylose into xylitol. However, the aim is also to remove xylitol dehydrogenase (EC 1.1.1.9) in this bioconversion despite its reducing the cofactor NADP to NADPH which is required for the xylose reductase [4]. A separation of xylose reductase from xylitol dehydrogenase will consequently provide a potential increase in xylitol production.

A sample from the cell suspension was taken for sonication and afterwards the supernatant was diluted for a further ultracentrifugation process.

As for the use of Amicon-Ultra devices, a Nominal Molecular Weight Limit (NMWL) characterizes the membranes used in Amicon Ultra devices; that is, their ability to retain molecules above a special molecular weight, solutes with molecular weights close to the NMWL may be retained only partially. That which determines the percent of recovery is the nature of protein solute and the starting concentration factor.

According to the literature, the molecular weight of xylitol dehydrogenase is 63-87 kDa [15-17] whereas the xylose reductase is 36 kDa [18]. By using the Amicon-Ultra-50K NMWL device, it is supposed to retain xylitol dehydrogenase at the membrane and as a result, to recover xylose reductase at the bottom of the tube. The determinations of protein and the xylose reductase activity were made after centrifuging the suspension of disrupted cells as well as after further ultracentrifugation processes of each diluted samples. The activity of xylitol dehydrogenase was also determined to assure that the partial purification process was successful (Figure 1).

By comparing the XR activity before and after the ultracentrifugation procedure, the yields of partial purification of XR for 1:4, 1:5 and 1:10 dilutions were then, obtained (Table 1).

Table 1 Xylose reductase activities after the ultracentrifugation procedure. The total and the specific activities of xylose reductase before being diluted were respectively 3.17×10^{-2} U and 8.92×10^{-4} U/mg of protein.

Samples	Total activity, U	Specific Activity, U/mg of protein	Yield, %
Diluted to 1:4	1.45×10^{-2}	7.93×10^{-4}	45.6
Diluted to 1:5	2.04×10^{-2}	1.96×10^{-4}	64.5
Diluted to 1:10	2.13×10^{-4}	1.22×10^{-3}	67.0

The data show that the best dilution for obtaining xylose reductase was 1:10 (yield=67.0%). However, both the dilution of 1:5 and 1:10 showed a productive result (yield of 64.5% and 67.0% respectively). Except for the first supernatant obtained after disrupting the cells, the activity of xylitol dehydrogenase was not detected in any of the filtrated samples. The yields may have been influenced by the presence of other proteins and other small impurities with similar molecular weight present with xylose reductase, which may have prevented the enzyme from passing through the membrane. The higher yield might be obtainable if the supernatant were very diluted or pre-purified before ultracentrifugation. Nevertheless, the dilution of 1:5 (64.5 %) besides offering a similar yield of xylose reductase, it also presented the advantage of being more concentrated.

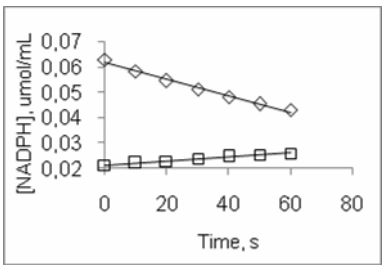

Fig.1 Profiles of xylose reductase activity {(◊) – 1.96 x 10^{-2} U, [NADPH] = -5.28x10^{-5}.t + 6.35x10^{-2}, R^2 = 0.998} and of xylitol dehydrogenase [(□) – 4.54 x 10^{-3} U, [NADPH] = - 9.41x10^{-6}.t + 1.21x10^{-2}, R^2 = 0.997] of *Candida guilliermondii*.

3.2 Conversion of xylose into xylitol

The batch assay was selected because of its simplicity as well as with a view for its future continuous application.

Through the G6PDH activities for both several NADP and G6P concentrations, the respective Lineweaver-Burk graphs were drawn, from which the correspondent K_M and V_{max} were calculated (Table 2).

Table 2. K_M values of XR [11] and G6PDH (from the baker's yeast, Sigma G-6378).

XR	K_M, µM	G6PDH	K_M, µM
NADPH	4.5	NADP	12.5
Xylose	6.4x10^4	G6P	2.97

Figure 2 shows the profile of the consumption of the mixture of sugars, which consisted of xylose and glucose-6-phosphate, catalyzed by both xylose reductase (XR) and glucose-6-phosphate dehydrogenase (G6PDH) enzymes. It can be seen from Figure 2 that after 1 hour of the reaction, 16.5% of the sugar was consumed whereas after 2 and 3 hours of the reaction, 15.2 and 35.5 % were consumed respectively.

Through this result, we may assume that up to the first hour, the main reaction was to attain a favorable concentration of NADPH for xylose reductase activity. At the interval from 1h to 2h the regeneration of cofactor reached the steady state leading to the consumption of both sugars. After 3h of process the yield of the xylose/xylitol conversion was about 36%.

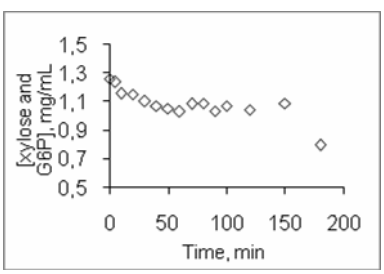

Fig. 2 Variation in conversion catalyzed by xylose reductase and glucose-6-phosphate dehydrogenase in a batch process.

An alternative to overcome the low yield in this procedure would be to concentrate the xylose reductase and to add a larger amount of glucose-6-phosphate dehydrogenase into the reaction. In addition, the latter attempt would also reduce the reaction time, which took 3 hours to obtain a yield of 35.5%. As Handumrongkul *et al.* [4] reported, the balanced utilization of cofactors dictates the rate of xylose metabolism in yeast.

This system is applicable due to the absence of both metabolic steps as well as the reduction of mass transfer limitations in this enzymatic procedure. For further purification processes, adopting the dilution of 1:5 (64.5 %) seems to be the best way to evaluate the conversion of xylose into xylitol by using the system made up of xylose reductase and glucose-6-phosphate dehydrogenase.

4. Conclusion

The use of both XR and G6PDH for the xylitol production reached a yield of 35.5%. The data showed that the best dilution for obtaining xylose reductase was 1:10 (67.2%). The usage of partially purified XR and G6PDH showed a promising way to improve the xylitol production.

Acknowledgements The support by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil) is gratefully acknowledged. The authors thank Philip Barsanti for his invaluable and constructive assessment of the manuscript.

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Production of recombinant forms of the propeptide COOH-terminal and the saposin B-type domain of the propeptide NH_2-terminal of the precursor of pulmonary surfactant protein B

A. Bañares-Hidalgo, E. J. Cabré, F. Gil, A. Bolaños, S. Aller[*], B. Amor[*], G. Castañer[*], A. Herráiz[*], V. Inaraja[*], M. Linares[*], L. Sanz[*], J. Pérez-Gil, P. Estrada[**]

Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense Ciudad Universitaria 28040 Madrid, Spain

[*]Undergraduate students

[**]Corresponding author: estrada@bbm1.ucm.es, Phone: +34 91 3944620, Fax: +34 91 3944672

The human surfactant protein B (SP-B) is a small protein produced by the proteolytic processing of the precursor proSP-B through elimination of two propeptides flanking the mature protein at its NH_2- and COOH-termini respectively. The sequences of the human propeptide COOH-terminal (SP-B_C) and the saposin B-type module (SP-B_{SAP-N}) of the propeptide NH_2-terminal have been cloned into the expression vector pMAL-c2x and sequenced. Expression of the fusion proteins MBP-SP-B_C and MBP-SP-B_{SAP-N} has been achieved in *E. coli* UT5600 cells after IPTG induction.

Keywords pulmonary surfactant, surfactant protein B, propeptide C-terminal, saposin B-type domain

1 Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins that spreads as a surface active film at the respiratory air-liquid interface, compensating physical forces tending to alveolar collapse. The surfactant protein B (SP-B) is essential to facilitate the formation of such a film. SP-B is a 79-aminoacid amphipathic protein produced by the proteolytic processing of a 381-amino acid precursor, proSP-B, which eliminates two propeptides flanking the mature protein at its NH_2- and COOH-termini respectively [1]. The propeptide of 177 residues flanking the NH_2-terminal side of the mature peptide is necessary and sufficient for targeting the processing and assembly of SP-B into surfactant [2].

SP-B is considered a member of the SAPLIP superfamily (saposin-like proteins) together with amoebapore, NK-lysin and saposins. ProSP-B is homologous to prosaposin, the precursor protein of saposins A, B, C and D [3]. ProSP-B contains three saposin B-type modules, including one in the mature protein and one in each of the flanking propeptides, and an additional saposin A-type domain within the NH_2-terminal propeptide. The possibility that the N-terminal and C-terminal saposin-like propeptides of proSP-B could have functional roles in the alveolar spaces once liberated from proSP-B has still to be explored. Other members of the SAPLIP superfamily, such as NK-lysin or the amoebapore [4], have antipathogenic activities.

The study of the conformational properties and the potential antipathogenic activities of the C-terminal propeptide of SP-B (SP-B_C) require the production of a recombinant form of the polypeptide, because the propeptide does not accumulate at the alveolar spaces once proSP-B is processed and this is our aim. Also we have intended the production of a recombinant form of the saposin B-type domain inside the NH_2-terminal propeptide (SP-B_{SAP-N}) in order to check whether the main features and properties of this propeptide, as previously characterized in our lab [5], are due to its saposin B-type domain. All the saposin-like proteins contain a well-defined pattern of intramolecular disulphide bonds not strictly required for the protein to acquire its functional conformation and full activity [6, 7] so that our aim has been the expression in *E. coli* of both proteins as a fusion with the Maltose Binding Protein (MBP) to facilitate its folding and solubility.

2 Materials and methods

Escherichia coli DH5αF´and UT5600 were used as host strains for plasmid production and protein expression respectively. Cells were maintained in LB containing 1% (w/v) bactotryptone (Sharlau), 0.5% (w/v) yeast extract (Scharlau) and 1% (w/v) NaCl supplemented with 100 μg/mL ampicillin (Ap) (Ecogen). Solid LB contained 1.5% agar (Scharlau). When needed, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Applichem) was included in the medium (50 μg/mL) as well as 4 μL of 800 mM isopropyl-β-D-thiogalactopyranoside (IPTG, from Genaxis). Vector pMAL-c2x (Fig. 1A) was purchased from New England Biolabs. The plasmid pENTR/D TOPO containing the sequence encoding for proSP$B_{\Delta C}$ (proSP-B lacking the

COOH-terminal propeptide) was a generous gift from Dr. T. Weaver (University of Cincinnati, USA) and was used as PCR template to amplify the sequence of the saposin B-type domain inside the amine terminal propeptide. The plasmid pET15b-preproSP-B containing the full preproSP-B sequence was also a gift of Dr. J. Johansson (University of Uppsala, Sweden) and was used as template for the PCR amplification of the COOH-terminal propeptide.

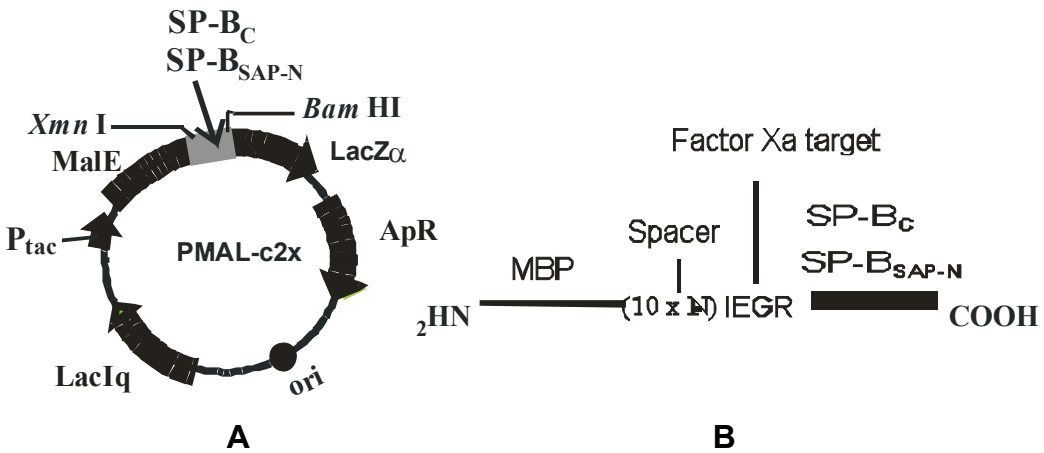

Fig. 1. (A) pMAL-c2x cloning vector containing malE gene which codifies the Maltose Binding Protein (MBP) of *E. coli* fused to the lacZα sequence through a polylinker, a spacer and the target for the Factor Xa protease. (B) Fusion protein scheme including the MBP polypeptide, the spacer with ten asparagines, the tetrapeptide target of Factor Xa and either the SP-B_C or the SP-B_{SAP-N} sequence.

Primers to obtain the gene sequence of the COOH-terminal flanking propeptide were designed based on reported sequence data (Genbank® Accession No. M24461) and are summarized in Table 1. The forward primer was coincident with the 5´ propeptide sequence while a stop codon (bold) and a *Bam*HI site (underlined) were incorporated in the reverse primer to facilitate subcloning in pMAL-c2x (Table 1). To amplify the saposin B-type domain (segment ^{59}V-Q^{147}) of the NH_2-terminal propeptide, the 5´end of the forward primer was coincident with the V^{59} codon whereas the 5´end of the reverse primer included a *Bam*HI (underlined) and a stop (bold) sequence prior to the Q^{147} codon (also in Table 1).

Table 1. Primer sequences for PCR amplification

Amplicon	Primer	Sequence
SP-B_C	Forward	5´-GATGACAGCGCTGGCCCAAGGTC
	Reverse	5´-CGGGATCC**TCA**AAGGTCGGGGCTGTGG
SP-B_{SAP-N}	Forward	5´-GTCTGGGGACATGTGGGAGCCGA
	Reverse	5´-CGGGATCC**TCA**CTGCCGGGATTTGCACAGG

PCR was carried out in a Master Cycler Personal (Eppendorf) with Vent Polymerase (New England Biolabs). Amplification conditions included 25 cycles of denaturation at 95 ºC (1 min), annealing at 55 ºC (2 min) and chain extension at 72 ºC (1 min). DNA fragments obtained were checked for size in agarose gel electrophoresis with Tris-Acetate-EDTA buffer, purified with organic solvents [8], digested with *Bam*HI and ligated (O/N, 16 ºC, T4 DNA ligase from Epicentre, in a water bath) with pMAL-c2x previously linearized with *Bam*HI/*Xmn*I.

Restriction endonucleases *Bam*HI (Amersham Pharmacia Biotech) and *Xmn*I (New England Biolabs) were used at 37 ºC for 6 h. Other conditions as prescribed by the supplier. Other DNA manipulations such as plasmids isolation were carried out by standard procedures. Two hundred µL of DH5αF´ competent cells prepared according to Hanahan [9] were transformed with 10 µL of the ligation product by the heat-shock procedure [8], including the addition of 1 mL SOB (2 % ,w/v bactotryptone, 0.5 %, w/v, yeast extract, 10 mM NaCl, 2.5 mM KCl) and 10 µL of 2 M glucose. After 1 h incubation at 37 ºC, 50 µL of the cells were platted on LB containing Ap, X-Gal and IPTG and incubated O/N at 37 ºC. Plasmids from putative recombinants were purified and the presence and orientation of the inserted DNA were determined by sequence analysis. Recombinant plasmids were used to transform competent UT5600 cells and the sequence was again assessed.

Cells transformed with pMAL-SP-B_C were grown at 37 ºC in 1 L LB cultures supplemented with 0.2 % glucose (w/v) at 150 rpm in an orbital incubator SI 50 (Stuart Sc.). When A_{600} = 0.5-0.7, IPTG was added to a

final concentration of 0.5 mM, cells were incubated for 60-75 min, harvested by centrifugation at 4000 x g for 20 min, suspended in 60 mL lysis buffer (20 mM Tris-HCl pH 7.4, 10 mM EDTA, 200 mM NaCl) and frozen O/N at –20 ºC. After thawing in cold water, a protease-inhibitor cocktail was added (Sigma) and 10 mM 2-mercaptoethanol (2-ME) to maintain reducing conditions. Cytoplasmic content was released by sonication using a Digital Sonifier 450 (Branson Ultrasonics Co.) equipped with a tip (20 % amplitude, 12 bursts of 10 s ON, 0.1 s OFF with 1 min pause in between, 4 ºC). Protein release was checked after every burst by mixing 5 µL of the sonicated fraction with 1.5 mL Bradford reagent [10] until a constant measure was reached and cell debris were removed by centrifugation (9000 x g , 30 min, 4 ºC). To measure protein in buffers containing 2-ME, the modification of Ross and Schatz [11] of the procedure of Lowry *et al.* [12] was employed. Otherwise, the Lowry method was used. Bovine serum albumin (SIGMA) was used as standard.

SDS-PAGE was performed as described by Laemmli [13]. Proteins were either dyed with Coomassie or electroblotted (*Western blotting*) onto Hyperfilm ECL membranes (Amersham Pharmacia Biotech). Membranes were blocked O/N with 0.1 % Tween 20 and 3 % (w/v) milk in PBS and incubated with monoclonal anti-MBP peroxidase conjugate (Sigma) at a final dilution 1/8000 for 90 min at room temperature. Alternatively, membranes were incubated with a primary monoclonal anti-proSP-B_C or anti-proSP-B_N antibodies (a gift of Dr. Weaver, University of Cincinnati, USA) to detect either MBP-SP-B_C or MBP-SP-$B_{SAP\text{-}N}$. They were used at 1/5000 and 1/2000 respectively and bound antibodies were probed with a secondary anti-Mouse IgG peroxidase-conjugate antibody (Sigma) at 1/1000. Blots were washed with PBS containing 0.1 % Tween 20, and proteins were visualized using enhanced chemi-luminescence detection kit (Amersham Pharmacia Biotech).

3 Results

We have cloned the C-terminal propeptide of proSP-B sequence in pMAL-c2x. The DNA obtained through PCR amplification was electrophoresed obtaining a fragment of about 300 bp (Fig. 2A, lane 1) which could fit with the expected size (306 bp without overhanging addition) of the amplified product. After ligation of the purified amplified fragment and linearized pMAL-c2x, transformation to DH5αF´ cells was carried out.
Individual colonies were screened for insert by duplicate streaking on Ap plates with (tester plate) or without (master plate) IPTG and X-Gal. Since putative recombinant clones do not form blue colonies on the tester plate, they were isolated and their plasmids purified.

Recombinant vectors were electrophoresed for checking size (Fig. 2C, lane 1) showing a reduced mobility in the gel compared with the non recombinant vectors (Fig. 2C, lane 2). After the recombinant DNA sequence was assessed, transformation of UT5600 cells was carried out and the nucleotide sequence of four recombinant clones was confirmed so that we proceeded to protein production. On the other hand, the sequence coding for the saposin-like domain of the NH_2-terminal propeptide was also amplified by PCR as shown in lane 1 of Fig. 2B. The mobility of the non recombinant and recombinant plasmids is shown in lanes 1 and 2 respectively of Fig. 2D.

Once the sequence of the fragments cloned in pMAL-c2x in UT5600 strain was assessed, we induced the recombinant protein expression with IPTG followed by bacterial harvesting, cell lysis by sonication and soluble fraction recovery after centrifugation. Aliquots of the soluble fraction mixed with loading buffer were subjected to SDS electrophoresis and electroblotted. Detection of the fusion proteins MBP-SP-B_C and MBP-SP-$B_{SAP\text{-}N}$ was carried out with anti-MBP antibodies and is shown in Fig. 3A and 3B respectively, where one can also see the lack of signal of non induced controls. As positive controls we used the fusion proteins MBP-βGal and MBP-SP-B_N that we had previously isolated and purified and MBP released after Factor Xa (FXa) digestion of the fusion MBP-SP-B_N (Fig. 1B) as described [5].

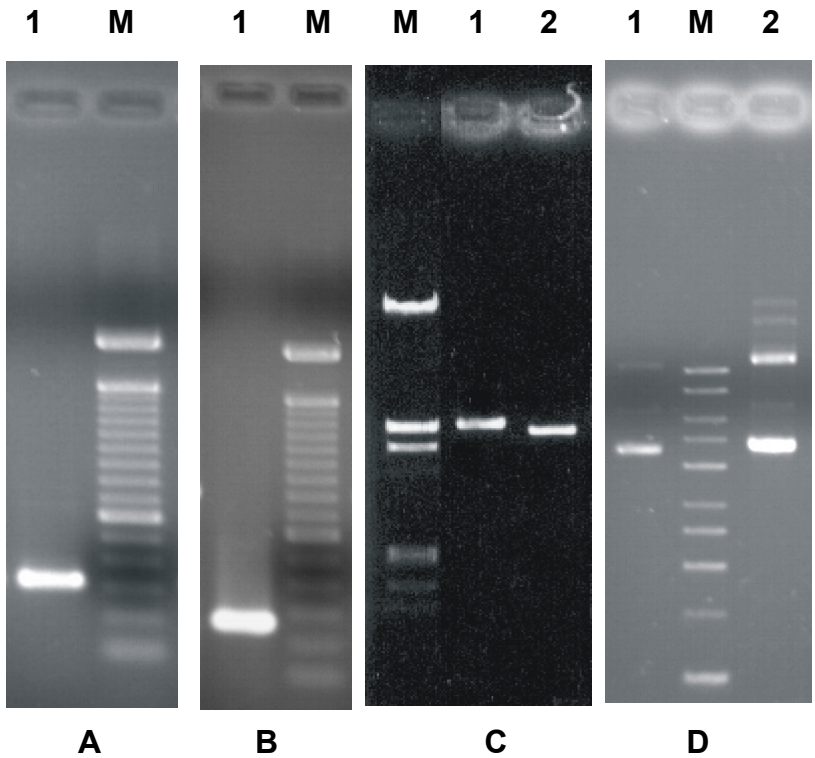

Fig. 2. Electrophoretical mobility of the PCR amplification product (A, B) and of the recombinant and non recombinant plasmids (C, D) in the way to clone SP-B_C (A, C) and SP-$B_{SAP\text{-}N}$ (B, D). Lane 1 in A is the SP-B_C PCR product (317 bp including the overhanging bases). Lane 1 in B is the SP-$B_{SAP\text{-}N}$ PCR product (278 bp including the overhanging bases). Lane 1 in C is pMAL-c2x-proSP-B_C (6943 bp) and lane 2 is pMAL-c2x (6646 bp). Lane 1 in D is pMAL-c2x (6646 bp) and lane 2 is pMAL-c2x-SP-$B_{SAP\text{-}N}$ (6904 bp). Molecular weight markers are meant M.

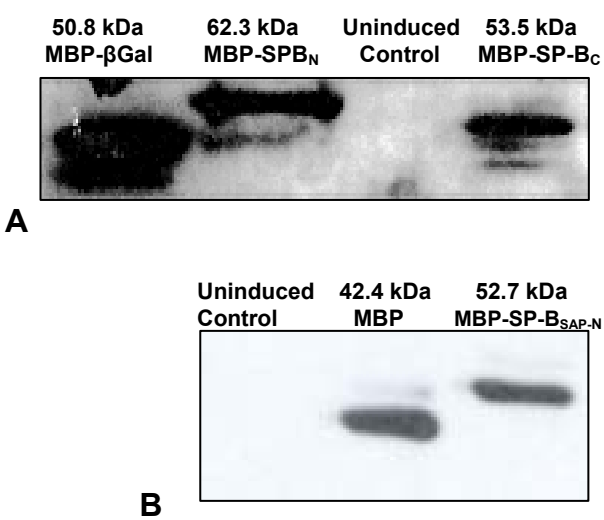

Fig. 3. Detection of the recombinant fusion proteins expressed in *E. coli* by SDS-PAGE and *Western blot* analysis with anti-MBP antibodies. In A, MBP-SP-B_C is MBP (Maltose Binding Protein) fused to the COOH terminal propeptide of the precursor proSP-B, MBP-SP-B_N is the fusion of MBP and the NH_2 terminal propeptide of the same precursor and MBP-βGal is MBP fused to β-galactosidase. MBP-SP-B_N and MBP-βGal were used as positive control. Un-induced culture was employed as negative control of MBP-SP-B_C expression. In B, MBP-SP-$B_{SAP\text{-}N}$ is the fusion of MBP and Saposin B-type domain within the NH_2 terminal propeptide of proSP-B and MBP is the recombinant Maltose Binding Protein used as positive control.

4 Discussion

Cloning in pMal-c2x has been our choice to produce the recombinant proteins SP-B_C and SP-B_{SAP-N}, since cleavage of the fusion proteins by protease FXa can release the polypeptide chains SP-B_C and SP-B_{SAP-N} from MBP without any added amino acid. We have previously employed this cloning strategy to obtain recombinant SP-B_N, which was purified and characterized by mass spectrometry and tryptic digestion [5]. Although SP-B_N was produced in *E. coli*, none of its ten cysteines was found to be free once released from MBP and moreover fluorescence studies indicate that the protein is folded (unpublished results). Interestingly, when FXa was neither inhibited nor removed after the cleavage of the fusion protein, the recombinant NH_2 terminal propeptide suffered additional non specific cleavages by FXa in the presence of low Ca^{2+} concentration, giving a polypeptide of about 15 kDa lacking the amine and carboxyl-termini but with the saposin B-type domain untouched [5]. We do not know yet whether this polypeptide is more or less coincident with the fragments of about the same molecular weight found either after the cleavage of the SP-B precursor *in vitro* by napsin A [14] or accompanying mature SP-B through Golgi and lamellar bodies *in vivo* [15].

Although we are interested in the behaviour of SP-B_N with respect to self-aggregation, oligomerization, interaction with lipids and antipathogenic activity, we intend to assess which part of this behaviour is due to its saposin B-type domain. To this, we cloned the SP-B_{SAP-N} sequence in the same expression vector (pMAL-c2x). Regarding the precise residues included into the saposin B-type domain there are minor differences with respect to its reported theoretical extension, involving either segment ^{60}W-P^{150} [3] or ^{65}A-S^{147} [16] so that we chose to clone the sequence ^{59}V-Q^{147} which included the border-line W^{60} (useful for fluorescence studies) flanked with the V^{59} residue to avoid the neighbouring of W to the FXa target sequence (IEGR) which could decrease the cleavage efficiency. This saposin B-type module of 89 amino acids has been expressed as a fusion protein with MBP as shown in Fig. 3B and its purification and characterization are now in progress.

Relative to the Carboxyl-terminal propeptide (SP-B_C), it has been proposed that cathepsin H is the protease responsible for its release *in vivo* from the precursor to yield the mature SP-B plus a small fragment of the NH_2-terminal propeptide along the secretory pathway [17]. Data from transgenic mice over-expressing this propeptide suggest a role of this module in the lipid turnover of surfactant [18]. So, our goal has also been to produce the MBP fused to the COOH-terminal flanking propeptide. The ulterior cleavage of this construct by FXa will allow the release of the soluble propeptide and its characterization in order to asses its functional role.

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Protease enzyme for surface degradation of wool fiber to improve dyeability

M. Parvinzadeh[*,1] and **M. Haji Ashrafi**[2]

[1] Young Researchers Club, Islamic Azad University, Shahre Rey Branch, Tehran, Iran
[2] Faculty of Postgraduate, Islamic Azad University, South Tehran Branch, Tehran, Iran

[*]Corresponding author: e-mail: mparvinzadeh@gmail.com, Phone: +98 9123137115

Nowadays, textile processing based on biotechnology have gained importance in view of stringent environmental and industrial safety conditions. The use of protease enzymes on protein fibers to improve some physical and mechanical properties is particularly interesting.
In this research, wool yarns were first treated with different concentrations of protease enzymes in water solution including 1%, 2%, 4% and 6% o.w.f. for 60 minutes. The dyeing process was then carried out on the treated yarns with pistachio hulls (50% o.w.f.). Some of physical, mechanical and colorimetric properties of treated wool yarns were discussed. Tensile strength of treated yarns was decreased due to enzyme treatment and it continued to decrease with an increase in enzyme concentration in solution. The lightness was decreased for the samples treated with enzyme. The wash and light fastness properties of samples were measured according to ISO 105-CO5 and Daylight ISO 105-BO1. The washing fastness properties of treated samples were not changed. In the case of light fastness properties, it was increased a little for 4% and 6% enzyme treated samples.

Keywords Wool; Enzyme; Fastness, Reflectance spectrophotometer

1. Introduction

In recent years, the use of low-environmental impact biotechnology giving rises to new types of treatment in the textile industry [1-3]. The best established application of biotechnology to textiles is the use of enzymes. These vital parts of all living organisms are organic catalysts with specific in the reaction catalyzed and substrates selectivity. Traditional chemical treatments are replaced by enzymes because of their lower product quality, higher manufacturing cost, more waste and added energy consumption. The main enzymes used in textile processing are amylases, cellulases, proteases, esterases, nitrilases, catalases, peroxidases, laccases and pectin-degrading enzymes [4-6].

A great many studies have been carried out on application of proteases, including antifelting and antishrink finishings, oxidative treatment followed by proteolysis, pilling performance, surface and appearance modification of wool fibers [7-10]. Makinson demonstrated that the hydrophobic properties and scaly structure of the wool surface are the main factors cause it's shrinkage. Proteases have been used for descaling process to improve shrink resistance [7]. The majority of enzymatic processes in the last few years have been combined with chemical pretreatments. In 1983, Kurashiki spinning Co. introduced a process for descaling the fibers using potassium permanganate as a pre-oxidising agent before proteolytic treatment [10]. Another experiments described that proteolytic enzyme improves the pilling behavior, the handle and luster of wool fabrics [10].

There have been relatively few papers studying the effect of protease on dyeing properties of wool with natural dyes. One study showed that protease produced a faster rate of dyeing with acid and premetalised dyestuffs and it decreased the apparent activation energy of dyes [9]. Other experimental evidence indicated that enzymes can be used as auxiliary agents in wool dyeing and they attack the surface of the fiber resulting more acid dyes absorption [10]. Tsatsaroni et al. reported the dyeing properties of two natural yellow pigments on protease treated wool fibers. Protease enzyme also tends to improve natural dyes absorption on wool fibers [10].

In the present study, wool is first scoured with detergent and then treated with enzymes at four different concentrations. The treated yarns are then dyed using pistachio hulls. The work focuses on the physical and chemical; properties of these dyed-enzyme treated wool yarns.

2. Experimental

2.1. Materials

The wool was Iranian yarn of 432/2 Tex with 144 twists/meter. The nonionic detergent used for the scouring of wool was obtained from Shirley Development Limited. The enzyme used was Novolan T from Novo Nordisk. It was produced by fermentation of genetically modified Bacillus microorganism. Acetic acid (85%) from Merck was applied for dyeing process. Powder of Iranian pistachio hulls was used for dyeing of protease treated wool yarns.

2.2. Procedures

Scouring: The Wool yarn was first scoured in 0.5% nonionic detergent for 30 min at 50ºC and a Liquor ratio of 40:1.
Enzymatic treatment: The enzymatic treatments were carried out for 1 h at pH 7 and 30ºC. Enzyme concentrations of 1, 2, 4 and 6% owf were used. The Liquor ratio was 10:1. Following the enzymatic treatment, the yarns were maintained for 5 min at a temperature of 90ºC and a pH lower than 4, in order to denature the enzyme. Finally, the yarns were rinsed to eliminate any remaining enzyme.
Dyeing: Dye solutions were prepared 24 h prior to dyeing by adding pistachio hulls powder to water (50% owf, liquor ratio 50:1). The dyeing process was started at 40˚C and the temperature was raised to 85˚C over 20 min and then held at that temperature for 1 h. The pH was maintained at 5 using acetic acid.
Some of physical, mechanical and colorimetric properties of treated wool yarns (dyebath exhaustion, Tensile strength, Reflectance measurement, Vertical wicking height and color fastness) were discussed. All measurements were repeated five times, together with standard deviation (the coefficient of variation was bellow 5% for all cases).
Determination of dyebath exhaustion: The effect of enzyme treatments on the percentage dyebath exhaustion was calculated according to Eqn 1:

$$E= (A_{\circ}-A_d)/ A_{\circ} \times 100 \quad (1)$$

Where $A_{\circ}$ and A_d are the absorbances (at λ_{max}) of dye originally in the dyebath and of residual dye in the dyebath respectively. The absorbances were measured by using a Shimadzu UV-2101 PC UV-Vis spectrophotometer.
Determination of Tensile strength: The tensile strength of the enzyme treated and untreated wool yarns was evaluated using an Instron TE-500 from Farayab with gauge of 20 cm and a cross-head speed of 25 cm/min, after conditioning the specimen for 24 h and 65% relative humidity and 20ºC (ASTM D2256).
Reflectance measurement: The reflectance of the dyed samples was recorded using a Gretagmacbeth COLOREYE 7000A spectrophotometer integrated with an IBM personal computer. CIELAB color coordinates (*L*, a*, b*, C** and *h*) were calculated from the reflectance data for 10ºC observer and illuminant D65.
Vertical wicking test: Effects of the enzyme solutions on the wicking properties of the yarns were also examined. For this purpose, samples were hung vertically in such a way that 1 cm of the sample soaked in the water container. After 1 min, the wet height of the samples was measured by means of a ruler. The samples were raw, scoured, and enzyme-treated (1, 2, 4 and 6%).
Determination of color fastness: The wash-fastness properties of the samples were measured according to ISO 105-C01. The color hue changes of the yarn and the degree of staining on the adjacent yarns were measured after drying. For light-fastness measurements, the yarns were exposed to the daylight for 2 and 7 days according to the daylight ISO 105-B01, and the changes in the color (fading) were assessed by the blue scale.

3. Results and Discussion

3.1 Dyebath exhaustions

Table 1 shows the exhaustion values for the untreated and enzyme treated pistachio hulls dyed wool yarns; Pistachio hulls showed much higher exhaustion on the sample treated with 1% enzyme solution comparing to untreated sample. An increase in the enzyme concentration resulted in an increase in the dyebath exhaustion. This confirms that protease catalyse degradation of epicuticle produce the fiber with more amine terminal groups, and as a consequence, improve susceptibility of dyes attraction. Further degradation is shown with increase in enzyme concentration [13, 16].

Table 1 Exhaustion of untreated and enzyme treated pistachio hulls dyed wool yarns (Standard deviations in parentheses)

Enzyme (%)	Exhaustion (%)
0	55 (1.2)
1	73 (1.5)
2	74 (1.7)
4	77 (1.6)
6	85 (1.5)

3.2 Vertical wicking test

Results of vertical wicking test on enzyme treated wool yarns are shown in Figure 1. It can be seen in Figure 1 that wicking height of raw wool increased with scouring; This means that the scouring process removes fiber fatty acids which are found in the cuticle surface. Enzyme treatment intensified the vertical wicking of scoured wool and its This confirms that protease catalyse rate was increased with any increase in the enzyme concentration. Cuticle provides hydrophobic barrier at the fiber surface while protease hydrolyses cuticle improving moisture diffusion from the surface into the fiber interior.

These results allowing the results obtained from the dyebath exhaustions.

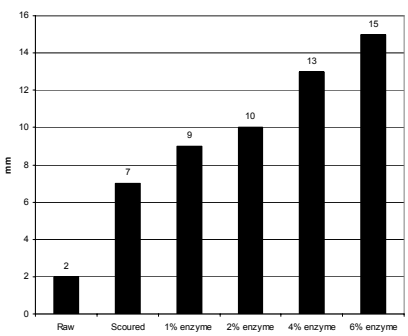

Fig 1 Vertical wicking height of raw, scoured, and treated wool with 1, 2, 4 and 6% enzyme (CV was less than 5%)

3.3 Tensile strength test

Table 2 shows that the modulus, tenacity and extension at break values for the sample treated with 1% enzyme were lower than untreated sample. A greater decrease in tenacity and extension at break can be seen by an increase in the enzyme concentration. It seems that enzyme penetrates into the fiber resulting degradation the cortical cells. Increase in enzyme concentration caused more degradation and structural damage which confirmed by Nolte et al.[10].

Table 2 Tensile properties of untreated and enzyme treated pistachio hulls dyed wool yarns (CV was less than 5%)

Enzyme (%)	Initial modulus (g/tex)	Extension at break (%)	Tenacity (g/tex)
0	28.3 (0.5)	31.5 (1.1)	6.23 (0.2)
1	25.1 (0.5)	28.4 (1.0)	5.42 (0.2)
2	23.8 (0.8)	27.5 (0.9)	4.83 (0.1)
4	22.9 (0.1)	25.08 (0.9)	4.81 (0.2)
6	20.4 (0.9)	22.04 (0.7)	3.77 (0.1)

3.4 Colorimetric measurements

The L^*, a^*, b^*, C^* and h values of enzyme treated pistachio hulls dyed wool yarns and untreated, are given in Table 3. The lightness (L^*) values showed decrease for the sample treated with 1% enzyme and decreased further as the enzyme concentration decreased. There was relatively small change in a^*, b^*, C^* and h for enzyme treated pistachio hulls dyed wool yarns. Decrease in L^* values could be the results of more dyes penetration into the enzyme treated wool yarns.

Table 3 Color co-ordinates of untreated and enzyme treated pistachio hulls dyed wool (Standard deviations in parentheses)

Enzyme (%)	L^*	a^*	b^*	C^*	h
0	33.15 (1.2)	2.98 (1.0)	25.07 (1.1)	27.02 (1.0)	58.32 (1.8)
1	30.88 (0.7)	3.56 (0.5)	25.88 (1.0)	28.77 (0.9)	59.50 (1.5)
2	30.55 (0.2)	3.69 (0.6)	28.13 (0.3)	28.06 (0.7)	57.20 (2.1)
4	29.07 (0.4)	3.05 (0.1)	28.02 (0.8)	28.50 (0.8)	56.39 (0.4)
6	28.23 (0.9)	3.35 (0.8)	29.93 (0.4)	28.00 (0.5)	57.06 (0.7)

3.5 Color fastness

The results obtained from the wash and light fastness tests are given in Tables 4 and 5. Enzymatic pretreatment does not affect the wash fastness for samples dyed with pistachio hulls.

After exposing the samples to daylight for 2 days, samples treated with 4 and 6% enzymes indicated better light fastness than others. Enzyme treatment caused to increase the dye aggregates into the fiber and the shades became darker. As a result, these aggregates are less vulnerable to the action of light.

With an extension of the duration of exposure to the daylight for 7 days, no more fading was observed. The color fading of the samples was limited to a certain period of exposure to daylight.

Table 4 Wash fastness properties of untreated and enzyme treated pistachio hulls dyed wool yarns

Enzyme (%)	Wash fastness	Staining on wool	Staining on cotton
0	4	4	4
1	4	4	4
2	4	4	4
4	4	4	4
6	4	4	4

Table 5 Light fastness properties of untreated and enzyme treated pistachio hulls dyed wool yarns

Enzyme (%)	After 2 days	After 7 days
0	3-4	3-4
1	3-4	3-4
2	3-4	3-4
4	4	4
6	4	4

4. Conclusion

Wool yarns were treated with 1, 2, 4 and 6% protease solutions for 1 h. Results of the UV–Vis spectrophotometry analysis on the remaining dyebath solutions showed that enzyme increased absorption of the pistachio hulls into the fiber. These results are similar to the results obtained from vertical wicking test. The color measurement tests confirmed that L^* values decreased with the treatment of enzyme, and also that any increase in the percentage of enzyme in the solution caused an increase in the amounts of L^*. There was no considerable change in wash-fastness test for enzyme treated pistachio hulls dyed wool yarns. In the case of light fastness properties, it was increased for samples treated with 4 and 6% enzymes. Protease hydrolyses hydrophobic barrier at the fiber surface improving dye absorption into the fiber interior.

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Subcellular forms of cholesterol oxidase from *Rhodococcus* sp. CIP 105 335: induction, solubilization and characterization

Aziz Elalami[1], Keith Baessler[2], Fanrong Kong[2], Nicole S. Sampson[2], Joseph Kreit[*,1]

[1] Department of Biology, Faculty of Sciences, P.O. Box 1014, Mohammed V University, Rabat, Morocco
[2] Department of Chemistry, Stony Brook University, Stony Brook, N.Y. 11794-3400, USA

[*] Corresponding author: e-mail: j.kreit@fsr.ac.ma, Phone: +212 37 77 54 61

Cholesterol oxidase (COX) induction in cells of *Rhodococcus* sp. (CIP 105335, strain GK1) depended on the sterol side chain. Octanoate, or Tween 80 showed a stimulating effect on enzyme synthesis by this strain. COX occurred in a secreted form and/or a cell surface-bound form depending on the carbon source used for strain growth. Extractability of the cell surface-linked form from cells with phosphate buffer or the buffer containing non-ionic detergent varied as the carbon source varied. The detergent-extracted COX was co-solubilized with mycolate and three proteins of around 52, 26, and 19 kDa. Some of these cell surface proteins might be involved in COX fixation into mycolate-rich cell surface. The secreted, buffer-extracted, and detergent-extracted enzyme forms showed the same substrate specificity and molecular mass, around 60 KDa. COX of GK1 is exported outside of the cell membrane, and either localized onto the cell surface, or released into the growth medium depending on the carbon source.

Keywords *Rhodococcus* sp.; cholesterol oxidase; induction; forms; characterization

1. Introduction

Cholesterol oxidase (COX) (EC 1.1.3.6) catalyzes the oxidation and isomerization of the sterol 3β-ol-5-ene structure into the corresponding 3-keto-4-ene-steroid with concomitant reduction of O_2 to H_2O_2. COX is produced by various microorganisms, including species of *Rhodococcus*, in a secreted form and/or cellular form. Induction of the enzyme and its secretion mechanism are not definitively understood. General reviews on the enzyme may be consulted for further information [1-3]. Throughout our investigation of COX, we have used a soil-isolated bacterium, *Rhodococcus* sp. GK1, which produces the enzyme in an extracellular form and a cell surface-linked form [4, 5]. A physiological approach, in which several steroids were examined as the sole carbon source of the strain GK1, showed that the induction of COX requires the C-17 side chain of sterols [6]. We continued the study of GK1 COX in order to understand better the enzyme induction, secretion, and extractability of the cell surface-linked form. The obtained data are described in the present paper.

2. Materials and Methods

Rhodococcus sp. GK1 (CIP 105335, Pasteur Institute Collection, Paris), a soil-isolated strain, was cultured in a medium composed of mineral salts [7], 0.8 mg L^{-1} thiamine-HCl, 3 $g.L^{-1}$ $(NH_4)_2$ SO_4 (basal medium), 4 $g.L^{-1}$ $CH_3COONa.3H_2O$ (1.7 $g.L^{-1}$ CH_3COO^-) and 2 $g.L^{-1}$ yeast extract; pH 7.0 (control medium). Steroids, octanoate (NaOH-neutralized solution) and Tween 80 (water-solution) were used each as enzyme biosynthesis effector. Steroids were sterilized as powders under UV. Growth was aerobic (medium occupying 1/5 of Erlenmeyer-flask volume) at 30°C with mechanical shaking (250 rpm for 100 ml culture). A seed culture was prepared in the control medium, and used to inoculate experimental cultures. For the study of the extractability or characterization of COX, GK1 was cultured in the basal medium (pH 7.0) containing variable carbon source; microbial growth was allowed in the above conditions. OD of cells was measured at 600 nm, and converted to dry cells (dc) using standard curves. Culture aliquots containing steroids were diluted in 2-propanol and 1% Triton X-100 solution (final concentrations: 10-20%, 0.5% w/v, respectively) to measure OD.

Acetate in growing cultures was determined by GPC: 0.25 ml sample filtrate was mixed with 0.05 ml of 4% methanol-0.75 N HCl solution. 2 µl mixture was analyzed using a chromatograph equipped with a Nukol column (modified polyethylene glycol/silica, 15 m x 0.55 mm id), flame ionization detector, and integrator. The oven temperature 180°C; the injector and detector temperatures 220°C; N_2 carrier gas was at an inlet pressure of 1 bar.

Steroids were extracted from HCl-acidified culture samples with chloroform. The solvent phase was filtered, evaporated at 60°C under a stream of air and the steroid residue was dissolved in 2-propanol. The concentrations of steroids with 4-en-3-one group were determined by measuring abs at 240 nm (ε_m = 1.40 x 10^4 $M^{-1}.cm^{-1}$); residual cholesterol or 5-androsten-3β-ol-17-one in these solutions were determined with an ABTS-assay [8].

TLC of steroids was performed on silica gel 60 plates, using a mixture of benzene and diethyl ether (3:2 v/v). Iodine vapor was sprayed on the plates to visualize steroid spots. Also, spot visualization was performed with sulfuric acid solution (50%) and heating. R_f of the steroids was: cholesterol 0.56; 4-cholesten-3-one 0.81; 5-androsten-3β-ol-17-one 0.39; 4-androsten-3,17-dione 0.52.

COX solutions were prepared as previously described [4]. When required, enzyme solutions were concentrated using evaporation under vacuum at 30°C, ultrafiltration on an Amicon membrane (under N_2 pressure of 1 bar) or a centrifugal ultrafiltration device. Cultures, cell suspensions and enzyme solutions were kept at 4°C, and used within one week, or stored at - 20°C for longer periods. COX was assayed at 30°C and pH 7.0 by monitoring H_2O_2 in a coupled assay [8]. The assay is compatible with whole cells. One enzyme unit (u) was defined as the amount producing 1 µmole of H_2O_2 per min. Specific activity was expressed in $u.mg^{-1}$ protein. Specific production of COX was expressed in $u.g^{-1}$ dc. Enzyme purification, SDS-PAGE, protein quantification, removal of Triton X-100 or Lubrol PX from enzyme samples and amount determination of these detergents were performed as previously reported [5]. Gel filtration of COX was performed on a column of Sepharose CL-6B (Pharmacia Fine Chemicals) or Sephacryl S-400 (Amersham Biosciences), using phosphate buffer (0.05 M pH 7.0) for column equilibration and elution. Unless otherwise specified, the chromatographed samples were detergent-free. Mycolate analysis was according to the procedure of Minnikin et al. [9] with mycolic acid of *Mycobacterium tuberculosis* (provided by Colorado State University) as reference.

3. Results

3.1 Cholesterol oxidase induction in cells of strain GK1

The steroids examined on the enzyme biosynthesis are two pairs of COX substrate and product that do (cholesterol, 4-cholesten-3-one), or do not (5-androsten-3β-ol-17-one, 4-androsten-3, 17-dione) have a C-17 side chain. Octanoic acid, which resembles to the cholesterol side chain, and Tween 80, an oleic acid ester, were also examined for their effect on COX synthesis. The strain was cultured in the control medium. Either steroid ($0.5\ g.L^{-1}$), octanoic acid ($1\ g.L^{-1}$) or Tween 80 ($1.5\ g.L^{-1}$) was added to the growing culture after 5 h of growth. Three independent replicates were performed for each assay.

The growth phase of GK1 in the control medium lasted 9 h with a generation time of 2.3 h ± 0.1. Complete depletion of acetate coincided with the end of this phase. The maximum biomass produced, expressed in g dc, was $0.70\ g.L^{-1} \pm 0.04$. COX was produced at a final level of $35\ u.g^{-1}$ dc ± 5 after about 15 h culturing. COX production was markedly weak during the growth phase of GK1, but maximal throughout the stationary phase after acetate depletion. The reason for this phase-dependent synthesis of COX is unknown. In any case, this enzyme production was not due to acetate effect, as it had been proposed previously [7]. A similar phase-dependent enzyme production was obtained from cells growing on acetate alone either at the same concentration as that of the control medium or at a higher concentration ($4.35\ g.L^{-1}\ CH_3COO^-$). Several sugars were used instead of acetate in order to formulate a control medium for microbial growth that would not provoke enzyme production. However, the strain grew slowly on glucose or ribose, and showed no capacity to metabolize sucrose or glycerol.

Cholesterol added into the microorganism culture was metabolized. Within 10 h post-addition, 90**%** of the initial sterol disappeared, and 40% of this sterol accumulated as products that absorb at 240 nm. Residual cholesterol and 4-cholesten-3-one as the main product were identified by TLC. After 37 h culturing (42 h total), cholesterol was present at a trace level and the accumulated derivatives absorbing at 240 nm, mainly 4-cholesten-3-one, was 30% of the initial cholesterol. The final additional growth, $0.16\ g.L^{-1}$ dc ± 0.02, was small and practically did not alter the growth profile on acetate-yeast extract (control). The use of 4-cholesten-3-one instead of cholesterol resulted in similar data for steroid catabolism and growth. Increase in the COX specific production became significant 4 h or 6 h after addition of cholesterol or 4-cholesten-3-one respectively, and maximal during the stationary growth, where 4-Cholesten-3-one was always present. The basic enzyme production on acetate-yeast extract had a mean value of $35\ u.g^{-1}$ dc and cholesterol or 4-cholesten-3-one induced a 1.9 fold or 1.7 fold (mean values) increase in enzyme specific production respectively.

4-Androsten-3, 17-dione was metabolized by the strain more rapidly than cholesterol or 4-cholesten-3-one. At 10 h post-addition, the additional biomass was $0.35\ g.L^{-1}$ dc ± 0.03. A similar level of growth was obtained with 5-androsten-3β-ol-17-one. There was no increase in enzyme specific production due to this sterol. In contrast, a notable repression of enzyme production was observed during the 10 h following its addition. This repression coincided with the accumulation of 4-androsten-3,17-dione that reached a maximum of 30% relative to the initial quantity of the 3β-hydroxy-steroid. 4-Androsten-3,17-dione completely repressed production of COX during the 10 h following its addition. Only, a partial recovery of enzyme synthesis occurred later, when the strain decomposed almost all of the keto-steroid.

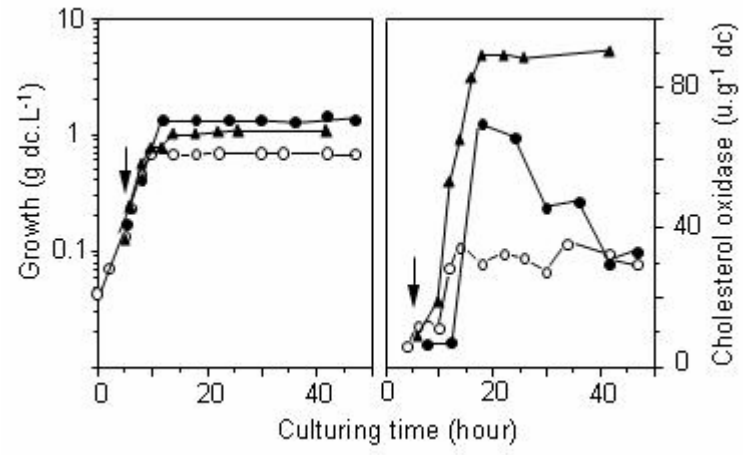

Fig. 1 *Effect of octanoic acid (●) or Tween 80 (an oleic acid ester) (▲) on cholesterol oxidase production by Rhodococcus sp. GK1*. Control (acetate + yeast extract) (○). Arrow, addition of octanoic acid or Tween 80. u, enzyme unit; dc, dry cells. The data shown are from a single experiment which is representative of three replicates.

The effect of *n*-octanoate or Tween 80 on COX production by GK1, cultured in the control medium, is shown in Fig. 1. The relative increase in the biomass was about 2 fold and 1.4 fold for octanoate and Tween 80 respectively. This of the enzyme specific production was at the maximum 2.0 and 2.6 fold.

3.2 Extractability of the cell surface-linked cholesterol oxidase

Enzyme extraction from cells grown on phytosterols (a mixture of sterols of which 56% is β-sitosterol), as the sole carbon source, was achieved using various non-ionic detergents [4]. An absolute requirement of detergent for this enzyme extraction was observed. Throughout the present study, it appeared that a fraction of the cell surface-bound enzyme was extracted by stirring cells in phosphate buffer, whenever the strain had been grown on a carbon source different from sterols, or on sterol together with another carbon compound. Besides, the enzyme extractability with detergent varied as a function of the microorganism carbon source. Representative data of the COX extractability with the buffer or the buffer-containing Triton X-100 are given in Table 1. The use of Lubrol PX, another non-ionic detergent, for COX extraction yielded similar results as with Triton.

3.3 Comparative characterization of cholesterol oxidase forms

3.3.1 Production

Data registered throughout the present study together with those reported previously [5, 6] showed that the level of the three forms of COX from the strain GK1 can be linked to the carbon source used for growth. Strain growth on cholesterol or phytosterols without any other carbon compound resulted in the production of the detergent-extractable form only. Additional carbon source, such as yeast-extract, cholate or acetate provoked the occurrence of the buffer-extractable form, the detergent-extractable form (cell surface-linked COX) together with the secreted form. The three forms were also obtained from cells gown on hexanoate.

3.3.2 Behavior throughout gel filtration and SDS-PAGE

A column of the gel Sepharose CL-6B (110 x 1.5 cm) was constructed, calibrated with protein markers (29-669 kDa) and used to examine behavior of the COX forms. In this study, the detergent-extracted form originated from cells grown on phytosterols, and the buffer-form was from cells grown on phytosterols-yeast extract. Samples of the enzyme forms were used at their crude extract state. Behavior of the extracellular COX on the gel had been described [5].

The buffer-extracted COX sample or the detergent-extracted COX sample (detergent-free) was fractionated on the column into a resolved activity peak and excluded activity peak. The relative elution volume (V_e/V_0) of the resolved activities corresponded to an apparent molecular mass of around 40 kDa and > 500 kDa for respectively the buffer-form and the detergent-form. These values were inconsistent with the molecular mass of the purified extracellular COX which was 59 kDa, as estimated by SDS-PAGE [5].

Table 1 Extractability of the cell surface-linked cholesterol oxidase (CSL COX) from *Rhodococcus* sp. CIP 105335 (strain GK1). Cells from the first stage of the stationary growth were suspended in 0.05 M phosphate buffer pH 7.0 [4], and enzyme was extracted by stirring the suspension magnetically at 20°C for 30 min. Enzyme solution was then collected by centrifugation. This enzyme extraction mode was repeated as indicated (extraction number). Enzymatic activity was measured for the cell suspensions (100%) and the collected extracts.

Carbon source ($g.L^{-1}$)	CSL COX ($u.g^{-1}$ dc)	Extraction number		Protein ($mg.ml^{-1}$)	SA[a] ($u.mg^{-1}$)	Extracted enzyme (%)
		buffer	detergent			
Phytosterols (a mixture of sterols	108	1		0.35	0.05	1
of which 56% is β-sitosterol) (5)			1	1.03	1.47	85
			2	0.58	0.34	11
Phytosterols (5)-yeast extract (2)	107					
Exp. 1		1		0.47	0.41	10
		2		0.29	0.39	6
			1	0.67	1.37	53
Exp. 2						
			1	1.10	0.94	61
			2	0.66	0.34	12
Cholesterol (5)	45	1		0.36	0.05	2
			1	0.88	0.65	71
Cholesterol (5)-yeast extract (2)	75					
Exp. 1		1		0.42	0.29	9
		2		0.30	0.22	5
			1	0.74	0.86	52
Exp. 2						
			1	1.00	0.69	56
			2	0.58	0.21	10
Phytosterols (2.4)-cholate[b] (3)	71					
Exp. 1		1		0.40	0.46	15
		2		0.32	0.30	8
			1	0.70	0.69	45
Exp.2						
			1	1.06	0.53	52
			2	0.62	0.22	12
Hexanoate (1.86)	46					
Exp. 1		1		0.25	0.57	18
		2		0.18	0.35	8
			1	0.66	0.50	46
Exp. 2						
			1	0.80	0.49	55
			2	0.40	0.18	10
Propionate (3.8)	14					
Exp. 1		1		0.26	0.86	90
Exp. 2			1	0.75	0.30	95

[a] S.P., specific activity. [b] Cholic acid was a good carbon source for the strain. It had no extracting effect on cholesterol oxidase.

Conversion of the excluded activity of the buffer-form to the resolved activity was achieved by stirring (30 min, 20 °C) with 2 M urea or 0.5% (w/v) Lubrol PX, followed by chromatography on the gel column. Urea treatment caused a 10% loss in the sample activity. Behavior of the buffer-form throughout the gel was generally similar to that of the secreted COX form, but different of that of the detergent-extracted COX. By the gel filtration, there was no separation of the detergent-extracted COX from substances that coextracted with it. Urea or surfactant treatment of the excluded or resolved activity, prior to rechromatography, was inefficient at dissociation of COX from the coextracted substances. All the above observations were confirmed using a column of Sephacryl S-400.

The data by the gel permeation suggested that COX in the buffer-form sample was partly in an aggregate, while the enzyme in the detergent-extracted form was totally in aggregates, separable into two activity peaks. The physical nature of the aggregate in the buffer-form sample was different from that of the aggregates in the

detergent-form sample, because the former was dissociable with urea or surfactant, but the latter aggregates were not dissociable.

The buffer-form was purified by chromatography on a kieselguhr-cholesterol column. The specific activity of the purified sample was around 6 $u.mg^{-1}$, similar to that of the extracellular COX [5]. Purification of the detergent-extracted form was unsuccessful by this method, due to enzyme aggregation. This enzyme form did not purify by chromatography on a CM-Sephadex C-25 column, performed at pH 5.0 in the presence of 0.1% (w/v) Lubrol PX. Its isoelectric point should be similar to that of the extracellular form, pI 8.9 [5]. However, there was no separation of COX from the coextracted substances, and the whole sample was eluted in the void volume of the column.

Electrophoresis of the purified buffer-form (Fig. 2, a) resolved a major band of around 60 kDa. Four major bands were resolved from a crude sample of the detergent-extracted COX by electrophoresis (Fig. 2 b). The first band of around 60 kDa was assumed to be COX. None of the other bands (named cell surface proteins, CSPs), which were around 52, 26 and 19 kDa, can be the detergent-extracted COX, because the molecular mass of this form must be comparable to that of the secreted form or the buffer form. The CSPs were inseparable from the enzyme by conventional gel filtration, as judged from SDS-PAGE data. In addition, mycolic acid was identified as a component (two main spots) of the detergent-extracted COX that did not separate from the enzyme by gel filtration. Most likely, the mycolate exists in the ester form as mono- and di-mycolate trehalose, a cell surface structure common to rhodococci and mycobacteria.

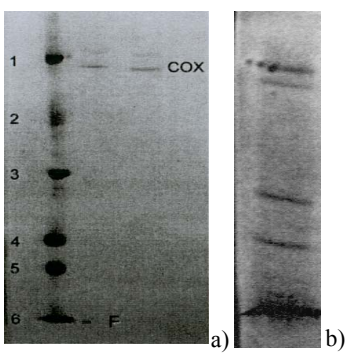

Fig. 2 *SDS-polyacrylamide gel electrophoresis of cholesterol oxidase (COX).* a) Protein markers (kDa): 1, bovine serum albumin 66; 2, ovalbumin 45; 3, glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) 36; 4, carbonic anhydrase (bovine erythrocyte) 29; 5, trypsinogen (bovine pancreas) 24; 6, trypsin inhibitor (soya bean) 20; F, migration front. Buffer extracted form (lanes 2-3): deposited protein amount was 5-8 μg of a purified sample. b) Crude sample of the detergent-extracted form: an amount of 40 μg protein was deposited; first band, COX; the other three bands, cell surface proteins (CSPs). The electrophoresis was run independently of a, using the same protein markers (not shown).

3.3.3 Catalytic properties

The secreted-form and the detergent-form of COX have similar substrate specificities [4, 5]. The buffer-form, identified in this work, was shown to share the same substrate specificity. Briefly, the enzyme forms are only active on the 3β-ol configuration of sterols, carrying out the stoichiometric formation of 3-keto-4-en-steroids and hydrogen peroxide. The sterol C-5 double bond is not necessary for the oxidation activity. However, the oxidation activity is increased by the presence of the C-5 double bond and/or the C-17 side chain of the sterol.

Because Triton X-100 is used in the COX assay to dissolve the enzyme substrate, the effect of this detergent on the buffer-extracted form was examined. Triton X-100 at concentrations that are around the critical micelle concentration (0.02-0.04%, w/v) caused a partial inhibition (60%) of the buffer-form activity. This inhibition, which had been seen previously for the detergent-extracted form of COX [4], was in the transition zone at the concentrations at which detergent micelles start to form. At these concentrations, unstable mixed micelles of detergent and cholesterol form shifting the free energy of the medium. As a result, substrate catalysis is thermodynamically lowered. The activity of the buffer-form just like the detergent-form or the extracellular form was stimulated by the detergent at concentrations higher than 0.05% and become maximal at 0.1 to 0.2 % detergent.

4. Discussion

4.1. Cholesterol oxidase induction

Cholesterol oxidase reaction is the first and compulsory step in the degradation pathway of sterols by rhodococci. Following it, these microorganisms proceed to degrade the sterol side chain and the sterol ring system independently. So that the limiting step in the side chain cleavage of cholesterol is the production of 4-cholesten-3-one. In the conditions of growth in the control medium, the weak catabolism of the cholestane

molecules by the strain GK1 may be due to an inhibition of the side chain cleavage, caused by some component of the yeast extract, as was reported for *Nocardia rhodochrous* (*Rhodococus rhodochrous*) [10].

Since 4-cholesten-3-one was present in the growth medium of GK1 during the culturing time (37 h), the additional COX production was probably due to induction by this steroid, or in the case of cholesterol by this ketosteroid together with the remaining sterol. Confirmation of the enzyme induction was obtained by addition of the transcription inhibitor, rifampicin (50 $mg.L^{-1}$), at 7 h following 4-cholesten-3-one addition. The antibiotic decreased enzyme production to a level lower than that of the control, demonstrating that the additional COX observed in the experiment was due to a *de-novo* protein synthesis. 5-Androsten-3β-ol-17-one was not a positive effector of COX production, although it is an enzyme substrate. 4-Androsten-3,17-dione, the product of the COX reaction on this substrate, repressed enzyme production. This result showed that there is no relationship between the catabolism of the steroid ring system and COX induction. Moreover, if the effect of the androstane couple is compared to that of the cholestane couple, the requirement for the C-17 side chain for COX induction could be identified, since these steroid couples are structurally different only by this side chain. This observation was in agreement with that in earlier studies [6], in which several steroids were examined both as the sole carbon sources of the strain, and as enzyme synthesis effectors. In the present study, these parameters were separated from each other, as the strain growth was mainly on acetate-yeast extract, and the steroids were each used in a small amount as enzyme synthesis effector.

The stimulating effect of octanoic acid on COX production by the strain GK1 could be linked to the similarity of the acid with the sterol side chain, as was the case with hexanoic acid [4]. In fact, the sterol side chain and fatty acid structures are alkanes, hydrophobic and both are catabolized by rhodococci throughout the β-oxidation pathway. Tween 80 is the ester of polyoxyethylene-sorbitan and oleic acid. The positive effect of Tween on COX production by the strain GK1 might be related to the acid moiety. A stimulating effect of oleic acid on COX synthesis by *Schisophyllum commune* was linked to the power of the fungus cells to adsorb the acid [11]. This effect of oleic acid was confirmed with COX production by the strain GK1 cultured in the control medium. Whether the mechanism of fatty acid effect on COX production in GK1 cells is by hydrophobic adsorption and/or by another way, is yet to be determined.

4.2 Occurrence of cholesterol oxidase forms

The variable extractability of COX from cells, observed as a function of the carbon source (Table 1), suggested a difference in the linkage topology of the enzyme molecule onto the cell surface. A carbon source effect on COX linkage is plausible, because the microorganism has to adapt in order to transport this source. Such an adaptation affects the cell surface structure, which in turn contributes to COX binding to the cell surface. The rhodococci capacity to adapt was well documented [12, 13], and the idea that adaptation of the present strain actually occurred was supported by the variations in the cell morphology and in the cells ability to aggregate, all as a function of the carbon source. Cells grown on sterols, that are hydrophobic molecules, were the most aggregated compared to those grown on other carbon sources. This cell aggregation may be due to an adjustment in cell surface topology and an accompanying increase in hydrophobicity that makes the cells compatible with the environmental molecules, in order to transport them. Consequently, the site of COX localization, where it is tightly bound, must be compatible with the surface hydrophibicity. This is in fact plausible, because solubilization of COX from cells grown on sterols absolutely requires non ionic detergent. Growth of cells on other carbon sources, less hydrophobic than the sterols or water-soluble (hexanoate, propionate, yeast extract, acetate), might result in a relative decrease in hydrophobicity of the localization site of COX. Consequently, the enzyme becomes partly extractable by stirring cells in the buffer without detergent. Furthermore, the relative diminution in hydrophobicity of the COX linkage in the cell surface might cause a relative release of COX into the medium during cells growth. Thus, enzyme secretion could occur. This statement can be firmly supported by consideration of COX production from cells grown on phytosterols, on phytosterols-yeast extract, or propionate. The enzyme total production was respectively: around 400 $u.L^{-1}$ (110 $u.g^{-1}$ dc), around 720 $u.L^{-1}$ (189 $u.g^{-1}$ dc), around 80 $u.L^{-1}$ (80 $u.g^{-1}$ dc). The enzyme secretion level was respectively: absent, around 45%, around 85%. The buffer extractable amount (Table 1), relative to the cell surface-linked COX, was respectively: absent, 15% (2 extractions), 90% (1 extraction).

The data obtained from the gel permeation chromatography demonstrated the existence of COX samples in active aggregates. These of the cell-bound COX, extracted with detergent, were different from the aggregate of the buffer-extracted enzyme, or the secreted COX [5], as judged by their elution profile and by their dissociability with urea or detergent. Analysis of the former aggregates showed that they were composed of three main proteins, in addition to COX, and mycolate which was probably in the ester forms with trehalose. All of these components coextracted with the surfactant, and coeluted from the gel filtration columns. Their lack of separation from each other indicated a high propensity for aggregation. It is obvious that the oxidase molecule is located in a mycolate-rich cell surface. Regarding the function of the coextracted CSPs, this remains to be

investigated. Hypothetically, some of these proteins might be involved in the oxidase molecule fixation to its locate.

The data of the molecular masses of COX forms, as estimated on SDS-PAGE or on the gel filtration, suggested that COX behaves unconventionally throughout the gel permeation. By SDS-PAGE the detergent-form of COX has an apparent molecular mass around 60 kDa, similar to that of the purified extracellular form or the buffer form. Thus, there is no difference in the apparent molecular mass of COX forms that could be detected by electrophoresis. The value, around 60 kDa, was comparable to those determined for the extracellular or the cell-bound COXs from different microorganisms, which were 55 to 61 kDa [1, 3, 14]. All of these enzymes are described as monomeric molecules. The similarity of the COX forms in the apparent molecular masses, the catalytic properties and the kinetics of their specific production (not shown) suggested that they are forms of the same enzyme. As for the relative amounts of their production, this depends on the carbon source, used for strain growth.

In conclusion, COX of *Rhodococcus* sp. GK1 occurs in an extracellular form and a cell-bound form (buffer-extractable and detergent-extractable forms), depending on the carbon source used for growth. Some of the cell surface proteins and the mycolate complex may serve to immobilize the enzyme onto the cell surface. Structural alteration of the cell surface under the control of the carbon source may be one of the factors responsible for COX secretion or cell surface localization.

Acknowledgements The authors are grateful to the NATO Scientific Affairs Division for the financial support (reference: LST.CLG. 980121, N. Sampson, J. Kreit) of this work as well as the National Institutes of Health (R01HL53306, N. Sampson).

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Microfactories – Microbial Production of Chemicals and Pharmaceuticals

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Challenges attending upon studies on clavulanic acid production

C. P. Sousa[*,2], I.L. Cruz-Hernández[1,2] , A. Baptista-Neto[1], J.C. Teodoro[1], M. Cerri[1], S.C.A. Ortiz[1], T.L. Freitas[1], M.L.G.C. Araujo[1], A.C. Badino[1,2] and C.O.Hokka[1,2]

DEQ1 and PPG-Biotec2, UFSCar, Rodovia Washington Luis, km 235, CEP 13565-905 Sao Carlos, SP, Brazil

[*]Corresponding author: prokarya@ufscar.br Phone: 55 16 33518325

In nature, microorganisms produce tiny amounts of secondary metabolic products as a matter of survival. Genetic manipulations are used in industry to obtain strains that produce hundreds or thousands of times more than that produced by the originally isolated strain. These strain improvement traditionally employ mutagenesis followed by screening or selection. Clavulanic acid (CA), a potent beta-lactamase inhibitor, is produced by strains of *Streptomyces clavuligerus* in submerged cultures. The aim of this paper was to report the advances achieved so far in our laboratories, in terms of strain improvement and bioprocess optimization involving oxygen supply, fed-batch operation and air-lift bioreactor design. We also report on our research into bioactive substances in endophytic microorganisms.

Keywords Streptomyces clavuligerus; genetic improvement; bioprocess optimization; endophytic microorganisms; bioactive substances

1. Introduction

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all fermentation processes. Conventionally, strain improvement (Parekh et al., 2000) has been achieved through mutation, selection, or genetic recombination using creative screening procedures. In nature, microorganisms produce tiny amounts of secondary metabolic products as a matter of survival. Genetic manipulations are used in industry to obtain strains that produce hundreds or thousands of times more than that produced by the originally isolated strain. The progress in strain and bioprocess improvement has increased fermentation productivity and decreased costs tremendously. Clavulanic acid (CA), a potent beta-lactamase inhibitor, is produced by strains of *Streptomyces clavuligerus* in submerged cultures. *S. clavuligerus* is able to produce a wide variety of antibiotics and other secondary metabolites including various β-lactam antibiotics. β-lactam antibiotics, such as penicillin and cephalosporin, have been extensively used for the treatment of many infections caused by pathogenic bacteria and their industrial process optimization has been the subject of many works (Thykaer and Nielsen, 2003; Ives and Bushell, 1997). The aim of this paper was to report the improvement and bioprocess optimization of the production of CA by *S. clavuligerus*. It is also reported the studies on bioactive substances produced by endophytic microorganisms isolated from Brazilian tropical savannah plants and from marine sediments. The paper presents the genetically improvement of the wild strain by classical mutagenesis; development of the bioprocess for the production of clavulanic acid and the investigation of new biomolecules in endophitic microorganism.

2. Material and Methods

2.1. Microorganism and strains

The *Streptomyces clavuligerus* ATCC 27064 used throughout this work was stored in the form of vegetative cells (5.0 $g.L^{-1}$ dry weight) at -70° C in cryotubes, utilizing glycerol 20% (w/v).

2.2. Analytical methods

The concentration of CA was determined as described by Foulstone and Reading (1982) – the HPLC method, and by Bird *et al*. (1982) – the spectrophotometric method. The glycerol concentration was determined by HPLC using a Shodex KS 802 column, following the manufacturer's instructions. The total lipid content was determined according to Postma and Stroes (1968). All the samples were assayed in triplicate. The cell concentrations were determined by correlating a rheological parameter and the cell concentration, a method described by Baptista-Neto *et al.* (2005), or by the dry mass method.

2.3. Culture media and experimental conditions for mutant experiments

The seed medium (Reading and Cole, 1977) contained (in $g.L^{-1}$ distilled water): glycerol, 15.0; peptone, 10.0; malt extract, 10.0; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4.7H_2O$, 0.75; salt solution 1.0 $g.L^{-1}$ ($MnCl_2.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001); and 3-(N-morpholine) propanesulfonic acid (MOPS) buffer, 21 (100 mM) and its pH was 6.8. The inoculum medium had the same composition as the seed medium, except that the peptone, malt extract and yeast extract were replaced by hydrolyzed soybean protein and MOPS buffer was added. The production culture medium contained glycerol and hydrolyzed soybean protein (Baptista-Neto *et a.l,* 2003; Rosa *et al.*, 2005). The culture medium contained vegetable oil and soybean flour (Maranesi *et al.*, 2005; Ortiz *et al.*, 2007). A medium (Sánchez and Braña, 1996) was utilized for strain preservation and spore formation.

2.4. Mutagenic treatment and monitoring

Mutagenic assays were conducted following Kieser et al. (2000) classical protocol. *S. clavuligerus* ATCC 27064 was cultivated in SB medium (12 days/28º C). Spores (2.5×10^4 spores mL^{-1}) were removed from plates with PBS, and determined by Neubauer chamber counting. Next, 10 ml of the spore suspension were transferred to dishes, stirred by hand and subjected to UV irradiation (15.2×10^{-6} $W.mm^{-2}$) at a distance of 60 cm from the lamp. Decimal dilutions from irradiated samples were made and aliquots were incubated in SB (10 days/28º C/in the dark). According Cruz-Hernández *et al.* (2007), only 1% of survivors and 180 mutants were isolated and tested in an incubator to determine the CA production. The phenotypic characteristics of growing colonies were observed.

2.5. Fermentation apparatus and cultivation procedure

Cell suspensions from cryotubes (3.5 mL), with a cell concentration of about 5 g L^{-1}, were mixed with 50 mL of reactivation medium in a 500 mL Erlenmeyer flask and incubated in a rotary shaker at 28°C and 250 rpm for 24 h. Then, 5 mL were inoculated into 500 mL Erlenmeyer flasks with 45 mL of production medium and incubated in a rotary shaker at 28°C and 250 rpm for 24 h. The culture obtained from the shaker cultivation was then inoculated in several 500 mL Erlenmeyer flasks, making up 10% of the inoculum volume. Cultivations were made in an orbital shaker under the same conditions for 120–160 h. One flask was removed every 12 h for sample analysis (Maranesi *et al*., 2005). In the fermentation cultivation, batch, fed-batch and continuous operation were performed in a Bioflo III fermentor with a 4-L working volume. The entire content of the flasks was transferred to the fermentor containing the production medium, corresponding to 10% inoculum. All the cultivations were conducted at 28º C and the pH was automatically maintained at 6.8 ($\pm$ 0.1). Batch and fed-batch cultivation conditions are described by Teodoro *et al.* (2006). The continuous process with and without cell recycling was performed (Baptista-Neto *et al.*, 2005). Samples were taken from all the cultivations to determine cell, CA, glycerol and oil concentrations.

3. Results and Discussion

3.1. Selection of CA producing mutants

To obtain the CA productive mutants, the wild *S. clavuligerus* ATCC 27064 spores produced were irradiated with UV light (200 s), which yielded 1% of mutant survivors. This irradiation time was chosen considering the survival curve in UV light irradiation (Cruz-Hernández *et al*., 2007). After this, 180 mutants were isolated in a shaker and a description was made of the phenotypical characteristics. Mutants AC13a, AC17a and AC70, which presented better production of CA were then selected. Figure 1 presents the results obtained for the AC13a mutant batch culture. As can be seen, the glycerol substrate concentration, Cs, decreased more slowly, reaching a value of zero after 30 h of cultivation. The consistency index, K, reached a high value of 35 $dina.s^n.cm^{-2}$, indicating consistent cell growth. This behavior also was observed in an analysis of the dissolved oxygen concentration in the broth, OD, which decreased precisely when the K parameter increased. The CA maximum concentration, Cp, was approximately 350 mg. L^{-1}, i.e., about 1.2-fold higher than that obtained in the standard batch culture.

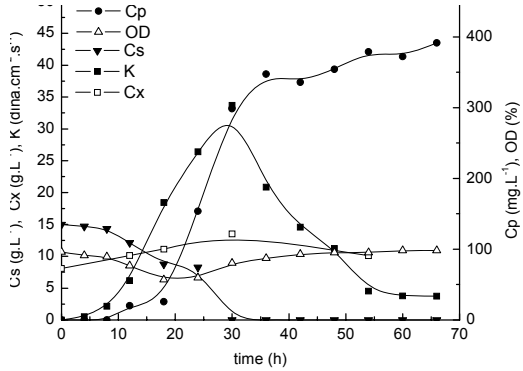
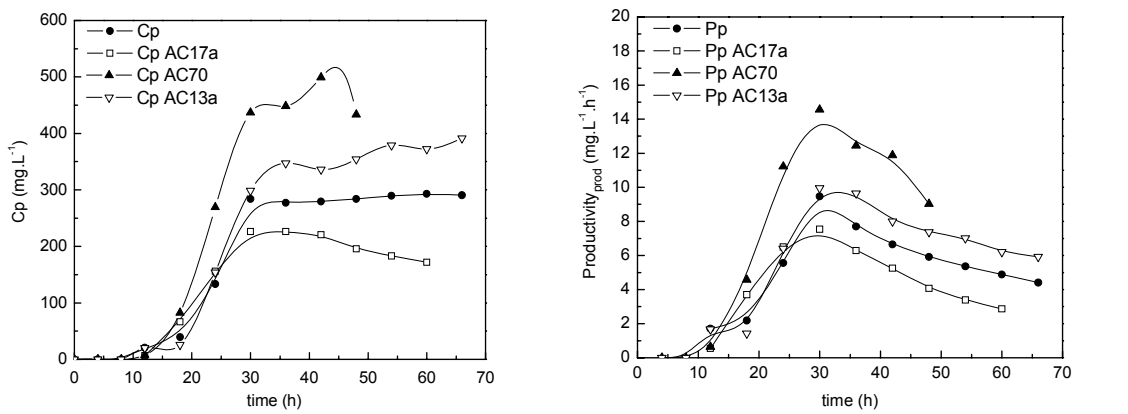


Figure 1 – Time course of cell, glycerol and clavulanic acid concentration in batch culture of mutant AC13a.

Figure 2 compares the maximum CA product concentration, Cp, and product productivity, Pp, for all the cultures. Figure 2a shows that mutants AC70 and AC13a produced a higher CA concentration than the standard culture, while the AC17a mutant showed some CA production capacity, but presented values below the standard culture. With regard to microorganism productivity, Cp/t, Figure 2b also indicates that the mutant AC70 presented the highest productivity, leading to the conclusion that this was the best mutant for CA production.



a) b)

Figure 2 – Comparison of the results obtained from all the cultures: (a) in terms of CA concentration, Cp, and (b) in terms of product productivity, Pp

3.2. Effect of dissolved oxygen and shear conditions on CA production

Bioprocesses using filamentous microorganisms frequently produce broths that display highly viscous pseudo plastic non-Newtonian behavior, affecting the bioreactor's performance (Cerri *et al*., 2005). Such is the case of CA production by *S. clavuligerus*. The cultivation broth presents an increasing apparent viscosity that can impair proper oxygen transfer during aerobic fermentations. This drawback can be partially offset by changing the operating conditions, namely, increments in impeller speed (N) and airflow rate, (Q), maintaining a proper oxygen mass transfer.

Bioprocesses were made on six batches in a 4-L conventional bioreactor utilizing a complex culture medium. Experiments were carried out at various stirrer speeds and different oxygen supply rates with controlled pH and temperature, and monitored or controlled DO. The results obtained were presented by Rosa *et al*. (2005), showing that shear rate positively affected CA production, contradicting the reports of other authors working on secondary metabolite production by *Streptomyces*.

Rosa *et al*. (2005) showed that the highest CA production was attained with a specific airflow rate of 0.5 vvm and under extreme shear conditions, i.e., 1000 rpm (v_{tip}=4.0 $m.s^{-1}$). The shear rate effect can be summarized (Fig. 3) by plotting experimental values of maximum CA concentration (C_{CAmax}) and maximum productivity (P_{CAmax}), which were obtained during the cultivations utilizing only the oxygen from the air, but under different shear conditions, i.e., v_{tip} of 1.2, 2.4, 3.2, 4.0 $m.s^{-1}$. These results show that CA production can be directly associated with shear conditions, represented by v_{tip}. It is worth mentioning that experiment involving low agitation speed combined with a high DO level (obtained by supplying enriched air) did not result in a higher CA yield than processes with high shear and non-enriched air.

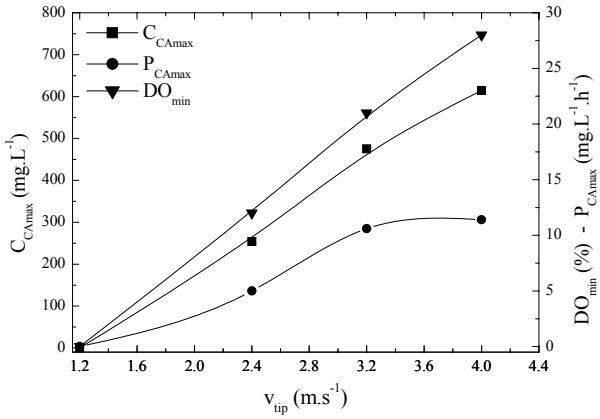


Figure 3 – Maximum concentration and volumetric productivity of clavulanic acid (C_{CAmax} and P_{CAmax}) and minimum dissolved oxygen concentration (DOmin) as a function of shear conditions.

3.3. Bioreactor operation mode

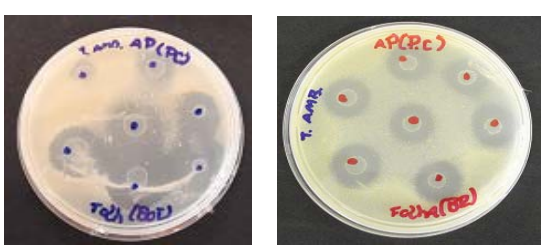
To study the effect of the bioreactor operation mode on CA productivity, experiments were conducted with bioreactors operating batchwise, fed-batch and continuously, with and without cell recycling. The data showed that the highest concentration was achieved when operating the process with the fed-batch reactor (700 mg. L^{-1}), as described by Teodoro *et al*. (2006). On the other hand, the continuous cultivation with cell recycling presented the best productivity (22.2 mg. L^{-1} h^{-1}), according to Baptista Neto *et al*. (2005).

3.4. Utilization of a pneumatic reactor – Airlift

Cerri *et al.* (2005) compared the cultivation of *S. clavuligerus* in airlift and conventional bioreactors. The cultivations were performed with the same oxygen transfer coefficients in both. The results showed that higher CA yields were obtained in the airlift bioreactor (442 mg. L^{-1}) than in the conventional one (404 mg.L^{-1}), demonstrating the advantage of the airlift reactor in CA production.

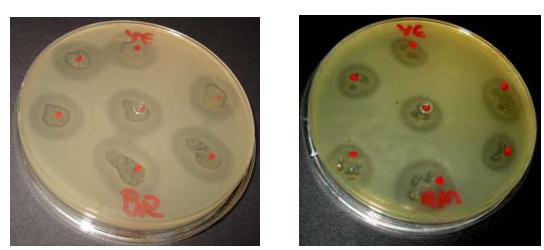
3.5. Results using endophytic microorganisms and antibiosis

Endophytic microorganisms have been relatively overlooked as potential sources of novel natural products for medical and commercial exploitation. It was investigated two Brazilian tropical savannah tree species, *Cassia leptophylla* and *Prunus* spp. Specimens were disinfected to eliminate the epiphytic population. Colonies were diluted and dispersed dropwise in media and growing colonies were inactivated. A *Staphylococcus* strain was used to the antibioses test. The resulting data indicated that the microorganisms isolated from *C. leptophylla* had no inhibitory effect on *Staphylococcus* (Ratti *et al*., 2008). On the other hand, microorganisms isolated from *Prunus* spp. Leave s showed inhibition against *Staphylococcus* (Ratti *et al*., 2008). The white colony showed antibacterial inhibition zones of 1.6 cm and 2.1 cm diameter in media when cultivated in PA and YE, respectively (Figure 4). Similarly, the other isolated microorganism (yellowish colony) presented inhibition zones of 2.0 cm and 1.4 cm diameter in media when cultivated in YE and PA, respectively (Figure 5).



(A) (B)

Figure 4 – The microorganism isolated from Prunus spp. (white colony) showed inhibition against Staphylococcus in PA (A) and YE (B).



(A) (B)

Figure 5 – The microorganism isolated from *Prunus* spp. (yellowish colony) showed inhibition against *Staphylococcus* in PA (A) and YE (B).

Acknowledgements. The authors acknowledge the financial support provided by FAPESP (Proc. 02/00189-1, 03/11722-5, 04/00224-7, 05/55079-4) and the scholarships granted by CNPq and CAPES.

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Growth and production of chitin and chitosan by *Syncephalastrum racemosum* using different carbon and nitrogen sources

A. C. Camarotti-Lima[1,2], J. B. Batista[3], M. C. F. Silva[1,3], R. V. S. Amorim[4], and G. M. Campos-Takaki[2]

[1] Northeast Biotechnology Network,
[2] NPCIAMB, Catholic University of Pernambuco, Boa Vista 50 050-590 Recife, PE, Brazil;
[3] FESVIP, Av. Rio Grande do Sul - 1169 - Bairro dos Estados, João Pessoa, PB, Brazil;
[4] Fungi Department, Federal University of Pernambuco, UFPE, 4Molecular Biology Department, Federal University of Paraiba, UFPB, Campus I – Cidade Universitária, 58051-900, João Pessoa, PB, Brazil.

The aims of this work were to investigate the growth of *Syncephalastrum racemosum*, Mucoralean fungus, and the production of chitin and chitosan using different carbon and nitrogen sources as cultures media. Sub-cultures on YPD plates of these strains were used to inoculate each condition to a final concentration of 107 spore's mL-1. The strain was grown at 28 ºC/150 rpm/96 h. The mycelium was harvested by vacuum filtration, washed with distilled water and freeze dried. Chitin and Chitosan were extracted by alkali-acid treatment. The results obtained with Peptone+Glucose medium produced the chitosan containing higher deacetilation degree, suggesting the best method. However, the best combination for increasing the amount of chitosan production by *S. racemosum* was obtained with Glucose+Glutamic acid medium, and suggesting the advantage of low cost associated to deacetilation degree.

Keywords *Syncephalastrum*, chitosan, chitin, serum of milk, glutamic acid

1. Introduction

Chitosan and chitin are polysaccharide polymers containing more than 5,000 glucosamine and acetylglucosamine units, respectively, and their molecular weights are over one million Daltons. Chitin is found in fungi, arthropods and marine invertebrates. Commercially, chitin is derived from the exoskeletons of crustaceans (shrimp, crab and other shellfish). Chitosan is obtained from chitin by a deacetilation process. Chitosan is an important constituent of fungi cells wall and, usually, the Zygomycetes Class shown higher amounts of chitin and chitosan in their cell walls when compared to other fungi [1, 2].

Despite of peptone meet extract served to requirement for nitrogen and carbon energy sources, fungi utilized a wide variety of carbon compounds aerobically [3]. *Syncephalastrum racemosum*, Mucorales order, class Zygomycetes, is studying as good representing of those class, because it has a high biotechnological potential to growth and source at chitin and chitosan in alternative and economic medium as sugar cane [4]. The present study aims to investigate the growth of *S. racemosum* and the production of chitin and chitosan using different carbon and nitrogen sources as cultures media.

2. Materials and Methods

2.1 Morphology Analysis

The microscopic morphology alterations in *S. racemosum* dependent of carbon and nitrogen source was analyzed by microcultivation in little blade per 14 days at 28ºC. All microcultures were with Aman blue and observed by optical microscopy at 400x.

2.2 Chitin and Chitosan Production, Extraction and Analysis

S. racemosum (UCP-00148) was obtained from Culture Collection, Catholic University of Pernambuco, Brazil. Sub-culture on YPD plates of these strains were used to inoculate in different carbon and nitrogen source (Table 1) to a final concentration of 107 spore's ml-1. Glucose concentration used as a unique carbon source was 2%, which suggest optimal for growth by aerobically or anaerobically condition. All culture was made in duplicate in Erlenmeyer flasks (250-mL) containing 100 mL of culture medium [3], with some modifications). The fungi strains were growth at 28 ºC/150 rpm/96 h. Mycelia strains were harvested by vaccum filtration washed with distilled water and freeze dried. Chitin and Chitosan were extracted by Synowiecki and Al-Khateeb [5], with

some modification. Washings procedures with deionizer water were done using filtration in Millipore membrane 200mm were used for purification of the polymers.

2.3 Chitosan characterization

The percentage of degree of acetylation (DA) was determined according to Roberts (1992) using the relative absorbance at wave numbers of (A1655/A3450)*100/1.33.

The infrared spectroscopy was using two milligrams sample of fungal chitosan, which had been dried overnight at 60ºC under reduced pressure were thoroughly blended with 100mg of KBr, to produce 0.5mm thick disks. Infrared spectrometer was recorded with a Bruker 66 Spectrometer, using a 100mg KBr disks for reference. The intensity of maximum absorption bands were determined by the baseline method.

2.4 Glucose, Nitrogen and pH determination

The carbon consumption was determined by the enzymatic colorimetric method (Labtest® Kit – Glucose oxidase). A standard curve was made by using a range of glucose solution (5 mg/mL). Colorimetric method Labtest® Kit for proteins was utilized for nitrogen consumption determination, using a spectrophotometer Spectronic Genesys 2. Changes in pH were measured using a potentiometer (Digital Potentiometer Quimis Mod. 400 A).

2.5 Statistic analysis

The data was analyzed for significance using the Student's t-test, dependent samples, using STATISTIC program version 6.0 of Statsoft Inc., USA. All experiments were carried out in quadruplicate.

3. Results and Discussion

3.1 Morphology analysis

Fungal morphogenesis was studied per different authors correlating with host invasion [6], environmental factors [7,8], and genetic keys [9, 10]. Our results did not shown morphology alterations dependent of carbon and nitrogen source in hyphae, columella, sporangium or sporangiospores (Figure 1-1S, 1-2S e 1-4Sa), although when *S. racemosum* was incubated in Serum milk+Glutamic acid culture media sporangiospore matured early (Figure 1-3Sa). In Glucose+Glutamic acid culture media, *S. racemosum* formed resistance spore (chlamydospores) (Figure 1-4Sb), suggesting these combination of carbon and nitrogen source is not a good promoted of *S. racemosum* grown, whatever glucose+glutamic acid culture medium is used to chitin deacetylase studies [11, 12].

3.2 Growth, Chitin and Chitosan Production

Aerobic cultures with different carbon and nitrogen source did not show an optimal pH ($p<0.05$), suggesting that differences in growth and chitin and chitosan production could be by another factor as medium composition and kind of microorganism [4, 12, 13, 14] (Table 1).

According to Bartcnicki-Garcia and Nickerson[3], *Mucor rouxii* showed an abundant growth when the fungus was incubated aerobically with defined basal medium added, individually, D-xylose, maltose, D-galactose, D-fructose, D-mannose and D-glucose as carbon source and when added, individually, L-glutamic acid or L-proline as nitrogen source. However, the use of lactose was not a good carbon source. In our experiments *S. racemosum* showed an abundant growth when growth in culture medium containing peptone and 2% of glucose. However, when the culture medium was supplemented with peptone at 10% from serum of milk, the growth was reduced ($p<0.05$). On the other hand, when was used 10% serum of milk in the culture medium with L-glutamic acid the *S. racemosum* did not showed an abundant growth, and the production of chitin and chitosan were reduced ($p<0.05$).

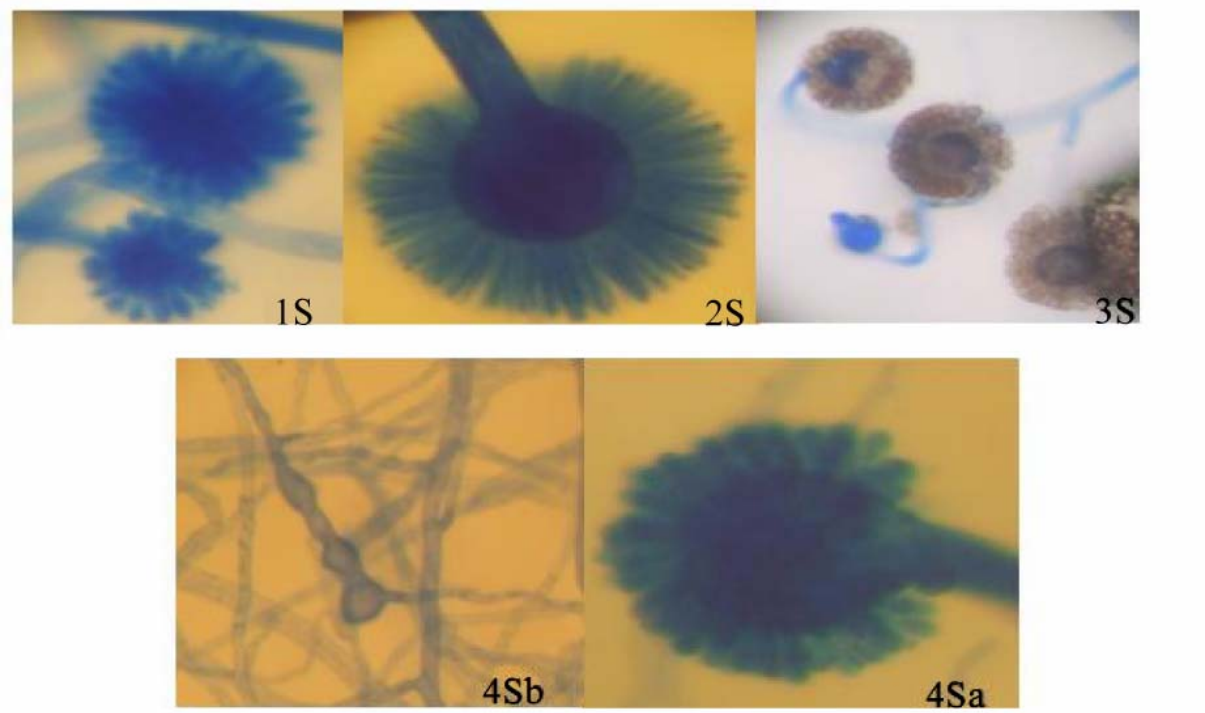

Figure 1. Photomicrographs at *S. racemosum*. No modifications dependent of carbon and nitrogen source of columella and sporangium, Peptone+Serum milk (1-1S), Peptone+Glucose (1-2S) e Glucose+Glutamic acid (1-4Sa). Resistent spore in Glucose+Glutamic acid (1-4Sb) and mature sporange in culture medium of Serum milk+Glutamic acid (1-3S). All figures are shown at the same magnification 400x.

Table 1 Different culture media. Growth and production of chitin and chitosan by *S. racemosum* and carbon and nitrogen source consumption influence after at 28 ºC/269 g/96 h. Biomass (mg dry wt/ 100 mL medium).

Conditions	pH	Biomass (mg)	Chitin%	Chitosan%	DA(%)	Carbon (mg/mL)	Nitrogen (mg/mL)
PEP (1%)+SERUM(10%)	8.29	234	16.33	2.65	52.89	0.0856	2.2128
PEP(1%)+GLUC(2%)	6.57	649	36.5	7.37	64.10	13.272	0.9021
SERUM(10%)+GLUT(1%)	8.52	170.5	10.42	3.66	58.82	0.0043	0.8511
GLUC(2%)+GLUT(1%)	6.41	12	33.56	56.64	54.62	10.5278	0.9957

Results are expressed as mean.
PEP = peptone extract; SERUM = serum milk; GLUC = glucose; GLUT = glutamic acid.

Despite the degradation of galactose being described by different authors to fungi [15, 16, 17]. The low carbon source consumption occurred when the milk serum was added to the medium with lactose [18], and it was not a good carbon source for *S. racemosum* ($p<0.05$) (Figure 2). The reduced production of chitosan and chitin is suggesting by inhibitory factors of chitin deacetylase in serum milk, as abundant Ca^{2+} or carboxylic acids [12].

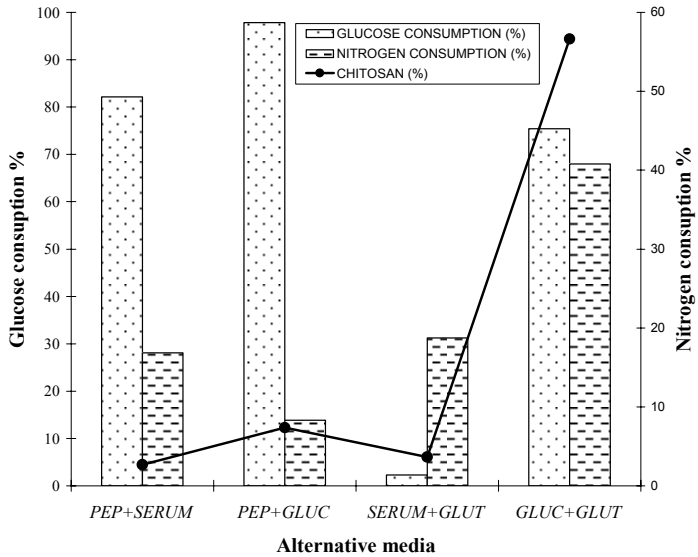

Figure 2. Carbon and Nitrogen source influence in chitosan yield.

Cultures incubated with glucose 2% added 1% of glutamic acid, as unique carbon and nitrogen source respectively, showed a significant reduced growth ($p<0.05$), although showed an abundant chitosan production ($p<0.05$) (Figure 3). Cultures incubated in basal medium add glucose and glutamic acid as carbon and nitrogen source, respectively, was used by different authors to chitin deacetylase studies [12, 13], suggesting these enzyme could be interfere with the higher yield of chitosan in Gluc+Glut alternative media. Another factor could interfere in the chitin deacetylase activity is pH culture media and was suggesting 4.5 for *S. racemosum* incubated in YPD medium (2% glucose + 0.3% yeast extract + 1% peptone) [19].

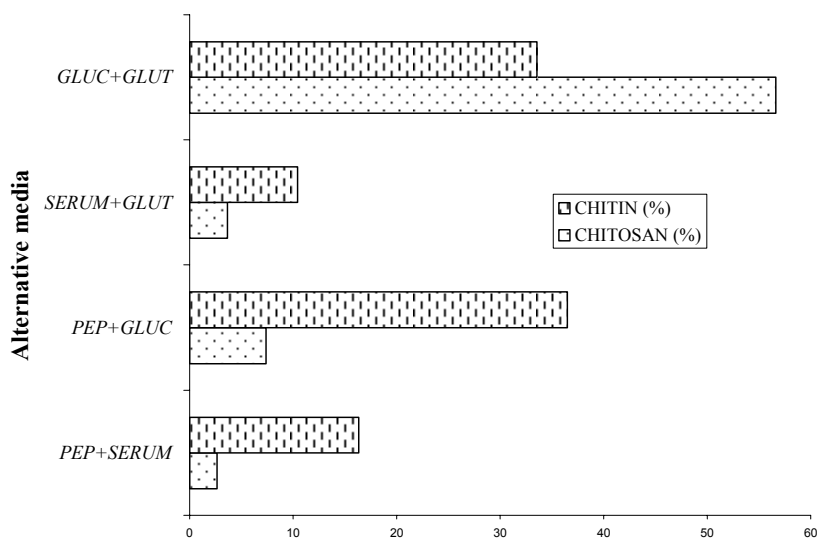

Figure 3. Alternative media influence in chitosan and chitin yield.

3.3 Chitosan analysis

Chitosan biosynthesis in fungi is suggested by tanden operating of chitin synthase and chitin deacetylase [20]. Characterization studies of chitin deacetylase using different microorganisms suggested modification in the pH, temperature, and some substrates for to obtain the optimal action [12, 13, 21, 22]. In our work chitosan production was not significant the parameters pH and temperature ($p<0.05$), but was dependent of carbon and nitrogen sources in culture medium ($p<0.05$) (Table 1).

Infra-Red analyse suggest no significant modifications on structure of chitosan dependent of carbon and nitrogen source to those shown by crustacean chitosan with the characteristics bands as the hydroxyl band at 3450 cm^{-1}, the amide band at 1655–1550 cm^{-1}, the amine band at 1630–1550 cm^{-1} and the C–H band at 3250 cm^{-1}.

In table 2, showed the comparative deacetylation degree (%), and suggesting when *S. racemosum* grown in alternative culture medium contained sugar cane juice as a good for chitosan production, and D-glucosamine content almost 70%. In our work th optimization process is progress to the deacetylation degree of almost chitosan produced when was used glucose (2%) added with glutamic acid (1%).

Table 2 Comparative D-glucosamine (%) from chitosan produced by the literature using alternative culture medium for growth

Organisms	D-glucosamine (%)	Alternatives culture media	References
Marine crustaceans	85.00	Standard	Sigma Pharma, USA
Syncephalastrum racemosum	54.62	Glucose+Glutamic acid	Our results
Syncephalastrum racemosum	75.00	Sugar cane juice	Amorim et al. [4]
Cunninghamella elegans	85.00	Yam bean	Stamford et al.[23]

4. Conclusion

The glucose used as carbon source in the medium having strongest influence on chitin and chitosan production. The chitosan produced by *S. racemosum* using Peptone+Glucose medium shown higher deacetilation degree and suggesting the best purification method for chitosan production. An important culture medium was Glucose+Glutamic acid containing the best combination of carbon/nitrogen sources for chitosan production in large scale. In addition, that culture medium showed facility for preparation and low cost of the components of the medium.

Acknowledgements The authors are grateful to financial support by CAPES, FINEP, CNPq and Catholic University of Pernambuco.

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Improving the carbon conversion rate in *Lactococcus lactis* fermentations: Cloning strategies

Maria Papagianni*, Nicholaos Avramidis, George Filiousis

Department of Hygiene and Technology of Food of Animal Origin, School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece.

*Corresponding author: e-mail address: mp2000@vet.auth.gr. Tel.: +30-2310-999804. Fax: +30-2310-999829.

The TCA cycle is a crucial link between catabolic and anabolic reactions in cells and anaplerotic reactions are responsible for replenishing the citric acid cycle intermediates. Extensive studies on the metabolism of an industrial *Aspergillus niger* strain have revealed that there are three main metabolic events that replenish TCA cycle intermediates and predispose the cell to product formation: fast uptake of glucose by simple diffusion, unrestricted metabolic flow through glycolysis, and uncoupled NADH re-oxidation resulting in lower levels of ATP and therefore decreased anabolic reactions. Only the activities of certain enzymes characteristic of individual *A. niger* strains can lead to such intracellular conditions. In this respect, we carried out studies aiming to demonstrate that the productivities and yields of industrial strains of *L. lactis* can be increased by reinforcing anaplerotic reactions by the transfer of specific *A. niger* genes into them. The development of a wide variety of cloning systems during the last decade has allowed the improvement of many properties of *L. lactis* strains that are essential for a large number of industrial dairy and other food fermentations. A central area in research related to *L. lactis* genetics is the regulation of glycolysis. The mechanisms responsible for regulation of the glycolytic flux and the shift between different fermentation modes are being studied extensively. Most recent research with microaerobic glucostat fed-batch experiments showed that the concentration of glucose influences its specific uptake rate and consequently the glycolytic flux, as well as the fermentation pattern. The highest specific activities of the key glycolytic enzymes PFK, PYK and the LDH were obtained at 55 mM glucose, the area of the highest observed glycolytic flux. Reduction of the glycolytic flux by 55% in the 277 mM glucostat corresponded to an almost identical reduction in PFK activity, indicating a certain controlling influence of this enzyme on the flux, through the glucose effect. Glucostat data also showed that the control of the flux through the glycolytic pathway under the examined conditions, resides to a large extent in processes outside the pathway, like the ATP consuming reactions and glucose transport. A regulation mechanism was proposed governed by the energy state of the cell by which *L. lactis* can handle the glycolytic flux through the allosteric properties of key enzymes, with PFK having a significant influence on the control. By cloning the *pfk*A and its truncated version alone and in strategic combinations with *aox*1 and *pka*C genes from *A. niger* into *L. lactis*, carbon conversion rates and the yield of lactate and nisin A were improved. *L. lactis* transformants performed more efficiently under aerobic conditions with high glucose levels in the medium.

Keywords *Lactococcus lactis*; cloning; carbon conversion

1. Introduction

Despite the impressive progress in *L. lactis* genetics and metabolism, we are still far from having a comprehensive understanding of central metabolic functions in this organism, such as the regulation of glycolysis, and the shift between different fermentation modes. Regulation of glycolysis of *L. lactis* has been extensively studied. Key glycolytic enzymes have been characterized and concentrations of glycolytic intermediates in cell extracts have been obtained in many cases since the eighties. Despite the wealth of metabolic information collected during years of intensive research and numerous genetic tools available for *L. lactis*, the fundamental question of what controls the glycolytic flux in this organism cannot yet be answered unambiguously [1].

LAB are characterized by numerous nutritional requirements. Several studies, in which different medium (defined or complex) formulations were used, have shown that glucose supports higher specific rates of growth, substrate consumption and product formation in *L. lactis* [2, 3]. Luesink et al. [4, 5] showed that growth of *L. lactis* on glucose resulted in higher activities of the glycolytic key enzymes phosphofructokinase (PFK), pyruvate kinase (PYK), and L-lactate dehydrogenase (LDH), the genes of which form the tricistronic las operon. Even et al. [3], using a novel DNA macroarray technology, showed that several genes of glycolysis were expressed to higher levels on glucose and that genes of the mixed acid pathway were expressed to higher levels on galactose. This explains the homolactic mode of fermentation supported by glucose [6, 7]. On the role of the PFK enzyme on the glycolytic flux in *L. lactis*, recent results reported by Andersen et al. [8] show that this enzyme plays an important role since glycolytic and lactate fluxes were decreased proportionally by a two-fold

reduction of PFK activity. A key role was also attributed to PFK with regard to the glycolytic flux control by Neves et al. [9].

Apart from the carbon source, aeration plays an important role in the metabolism of *L. lactis* and the cellular content of key enzymes changes with aeration. The negative effect of oxygen on expression of the *pfl* gene (encoding pyruvate formate lyase) is well known [10, 11], and the *pfl* gene is known to be very sensitive to oxygen [11]. Expression of the *adhE* gene (encoding alcohol dehydrogenase) is known to be reduced by aeration [12]. Moreover, the levels of the three key glycolytic enzymes were found to be lower in *L. lactis* cells grown under aerobic conditions [9]. In contrast, the in vitro specific activities of α-acetolactate synthase (ALS) and the pyruvate dehydrogenase (PDH) complex have been reported to increase with aeration [13, 14]. For the most part, *L. lactis* has been studied under totally anaerobic conditions or, in a few cases, under totally aerobic conditions [13, 15, 16]. Microaerobic conditions were applied in two studies performed by Jensen et al. [14] and Nordkvist et al. [7] both of them using glucose as the sole carbon source.

During the last decade, the development of a wide variety of cloning systems has allowed the improvement of many properties of *L. lactis* strains that are essential for a large number of industrial dairy and other food fermentations. A central area in research related to *L. lactis* genetics is the regulation of glycolysis. Application of metabolic control analysis revealed a number of factors that control the flux through glycolysis. Among other glycolytic enzymes, PFK seems to attract research interest since it has been shown to exert strong control over the concentration of upstream metabolites. Most recent research carried out by Papagianni et al. [17] with microaerobic glucostat fed-batch experiments in which glucose was maintained at stable levels (13.75 to 555 mM), showed that the concentration of glucose influences its specific uptake rate and consequently the glycolytic flux, as well as the fermentation pattern (homolactic vs mixed acids). The highest specific activities of the key glycolytic enzymes PFK, PYK and the LDH were obtained at 55 mM glucose, the area of the highest observed glycolytic flux, while reduction of the glycolytic flux by 55% in the 277 mM glucostat corresponded to an almost identical reduction in PFK activity, indicating a certain controlling influence of this enzyme on the flux, through the glucose effect. Glucostat data also showed that the control of the flux through the glycolytic pathway under the examined conditions, resides to a large extent in processes outside the pathway, like the ATP consuming reactions and glucose transport.

Extensive studies [18, 19, 20, 21] on the metabolism of an industrial *Aspergillus niger* strain, with close to theoretical efficiency in citric acid productivity, have revealed that there are three metabolic events that replenish TCA cycle intermediates and predispose the cell to product formation: fast uptake of glucose by simple diffusion, unrestricted metabolic flow through glycolysis, and uncoupled NADH re-oxidation resulting in lower levels of ATP and therefore decreased anabolic reactions. Only the activities of certain enzymes characteristic of individual *A. niger* strains can lead to such intracellular conditions. The TCA cycle is a crucial link between catabolic and anabolic reactions in cells and anaplerotic reactions are responsible for replenishing the citric acid cycle intermediates. Our work, in the frame of the ANTICO project – a 5th Framework Programme project co-financed by the European Commission- aimed to demonstrate that the productivities and yields of commercial microorganisms, among which *L. lactis*, that make a wide range of products can be increased by reinforcing anaplerotic reactions by the transfer of specific *A. niger* genes into them. By cloning the *pfk*A, *aox*1 and *pka*C genes from *A. niger* into *L. lactis* we intended to improve the yield of lactate and /or nisin A by enabling *Lactococcus* to perform more efficiently under aerobic conditions with high glucose levels in the medium. A summary of the activities undertaken towards this aim and the most important results are presented in this work.

2. Materials and methods

The activities undertaken were the following: 1)Transfer of *Aspergillus niger pfk*A gene coding for 6-phosphofructo-1-kinase into *L. lactis* strain alone, and in combination with *A. niger pka*C gene coding for catalytic subunit of camp dependent protein kinase; 2)Transfer of *A. niger aox*1 gene coding for alternative oxidase into *L. lactis*; 3)Transfer of both *pfk*A or *pfk*A-*pka*C and *aox*1 genes simultaneously into a recipient *L. lactis* strain; 4) Modifications of the transformed genes on the basis of reports obtained by metabolomics. Two cloning vectors were used throughout the project: the plasmids pVS44 and pTRKH3. Apart from *pfk*A gene, the truncated *pfk*13 was also cloned alone and in combinations with *aox*1 and *pka*C genes. For single, double or triple transformations, cassettes were constructed and genes were cloned behind the *pur*DEK promoter [22]. Two *L. lactis* strains were transformed with vectors containing *A. niger* genes: *L. lactis* ATCC11454 and *L. lactis* LM0230. An *Escherichia coli* strain (*E. coli* BL21) was also used as a cloning host for the vector plasmid pLN95 from which the *pur*DEK promoter was delivered. *Lactococcus* strains were routinely grown in M17 containing 0.5% glucose instead of lactose at 30^{o}C, in 150 mL Erlenmayer flasks, without agitation. *E. coli* BL21 was cultured at 37^{o}C in Luria-Bertani (LB) broth (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) or LB agar in 150 mL shake flasks at 200 rpm. Fermentation kinetics studies were carried out following

transformations for comparisons between parental and transformed strains. Biomass concentration, sugar consumption, lactic acid accumulation and nisin production were determined, as well as PFK and PKAC activities. Glycolytic enzyme activities were determined as described by Papagianni et al. [17]. PKAC activity determination was done according to Lübbehüsen et al. [23]. Nisin was determined according to Papagianni et al. [24]. Lactic acid concentration was determined with the EnzyPlus D/L Lactic Acid kit by Diffchamb AB Sweden.

3. Results and discussion

Transformants into which *A. niger* genes were cloned, alone or in combinations of two and three were constructed. Plasmid vector size reduction was performed prior to DNA cassettes cloning. The version without introns of *pfk*A, for cloning in bacteria, was called *pfk*A2. The *pur*DEK promoter was used for cloning *A. niger* genes into *L. lactis* (Kilstrup et al., 1998). The promoter was isolated from pLN95 vector (an *E. coli* / *L. lactis* vector resulted from modifications made on plasmid pAK80) into which it was fused to the *lac*LM gene. Promoter mapping was done by exonuclease III deletions. Cassettes were constructed and the following examples are given here for *pfk*A2 gene (Fig. 1) and the truncated *pfk* gene (*pfk*13) and the *aox*1 gene (Fig. 2):

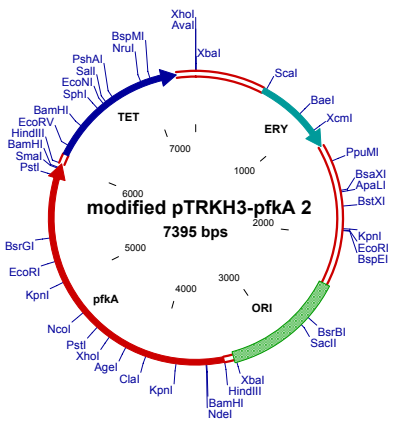

Fig. 1

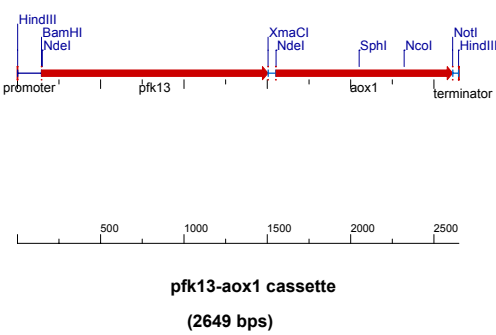

Fig. 2

Fig. 1: *pfk*A 2 gene cloned into modified pTRKH3 plasmid.

Fig. 2: Restriction analysis of the *pfk*13-*aox*1 cassette

A cloning vector containing a 3-genes DNA cassette is the following:

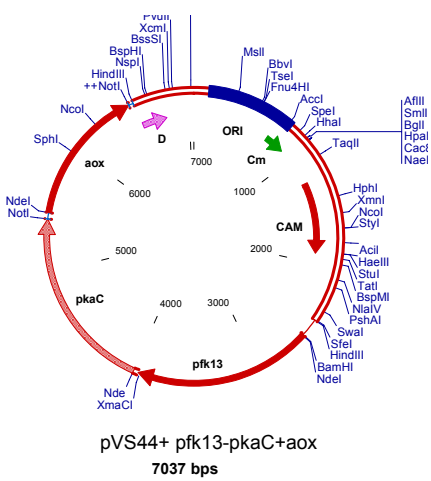

Fig. 3

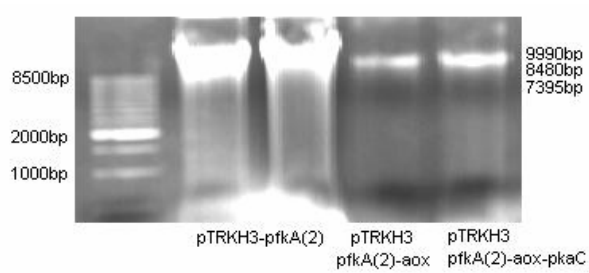

Fig. 4

Fig. 3: pVS44-based vector containing the *pfk13*, *pKa*C and *aox* genes from *A. niger*.

Fig. 4: Based on modified vector pTRKH3: *pfk*A, *pfk*A-*aox*1, *pfk*A-*aox*1-*pka*C containing plasmids

In a similar way, a number of plasmid vectors were constructed (Fig. 4) and electrotransformed into *L. lactis* strains. Trasformation efficiencies were in all cases in the order of 10^6 transformants /μg DNA. Both host-vector systems proved to be reproducible and highly efficient [25].

Fermentations were carried out in 10 L STR with various medium formulations. Results varied, however the strain with integrated t-*pfk*A-*pka*C-*aox* genes had significantly higher lactic acid productivity at double the carbon conversion efficiency (Fig. 5). On a complex medium with 40 g/l of glucose, the growth was more rapid and all the glucose was consumed within 10 hours. The t-*pfk*A-*pka*C-*aox* recombinant grew and used glucose more slowly than parental strain but had a higher glucose to lactic acid conversion efficiency and showed higher specific productivity.

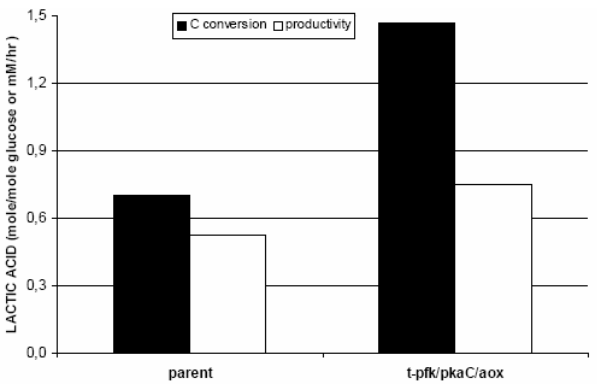

Fig. 5: C-conversion rates and productivities of parental *L. lactis* LM0230 and the triple transformant in defined media fermentations

PFK activity of the transformant containing *pfk*13, aox1, and *pka*C genes was estimated to be 750 units while PFK activity in the parental strain under the same experimental conditions did not exceed 300 units. Lantibiotic nisin production showed an impressive increase in the particular transformant: 9.000 IU/ml while only 2.000 IU/ml in the parental strain.

The main objective of this project was to demonstrate that the transfer of some strategic genes from *A. niger*, one of the most efficient industrial micro-organisms, into other commercially important micro-organisms will increase the productivity and/or yield of their bio-products. This was achieved by transferring specific *A. niger* genes that enhance anaplerotic pathways, the reactions that replenish tricarboxylic acid cycle intermediates, by virtue of their regulatory mechanisms. Increased productivity was evaluated apart from *L. lactis,* in *Aspergillus terreus*, *Pichia pastoris*, *Streptomyces rimosus*, *Streptomyces clavuligerus*. Double transformants of all recipient microorganisms have been prepared carrying either the native *A. niger pfk*A gene or truncated *pfk*A genes and aox gene. In transformants with a truncated *pfk*A gene that encoded the shorter *pfk*1 fragment, the *pka*C gene was introduced into the cells as well, enabling the synthesis of the catalytic subunit of cAMP-dependent protein kinase. The shorter fragment of *pfk*1 was synthesized in an inactive form that needed to be phosphorylated by a kinase to become active. In some recipient microorganisms such as *A. terreus* the *pka*C gene coding for catalytic subunit of cAMP dependent protein kinase (PKA) seemed to be lethal since no transformants could be isolated, no matter which method of gene transformation was used. Uncontrolled activity of *pka* could cause over phosphorylation of proteins in the cells which seemed to be harmful for cell metabolism. On the other hand in bacterial systems no increase in activity of *pfk*1 could be detected in transformants carrying truncated *pfk*A gene and *pka*C gene indicating that *A. niger pka*C gene could not be properly synthesized or folded to form an active enzyme.The research carried out throughout this project clearly showed that introducing specific key *A. niger* genes of the primary metabolism into various commercial micro-organisms increased the pool of precursors and improved their productivity or increased the yield of specific bio-products. The addition of heterologous genes didn't have the same effect in all recipient microorganisms but in the best cases the productivity was nearly doubled. In many transformants carrying *A. niger* genes significantly increased specific productivity was detected that was more often recorded with the strains expressing *pfk*A gene and was even more prominent with transformants carrying truncated *pfk*A gene normally in the combination with *pka*C gene. In *A. terreus,* for example, the addition of *A. niger* genes had a significant positive effect on itaconic acid levels, while no lovastatin production could be detected during the growth in a glucose rich medium, either with parental strain or with any of transformants, although the strain used should excrete lovastatin according to the data in the literature. Transformants with the inserted native *pfk*A gene, truncated *pfk*A gene and *aox* gene accumulated about 30% more itaconic acid at the end of fermentation than the parental strain, while double transformant

carrying truncated *pfk*A gene and *aox* gene also produced more acid. However no increased productivity could be detected with transformant harbouring the native *pfk*A and *aox* gene. Interestingly all transformants with any kind of *pfk*A gene added used glucose more efficiently, while *aox* mutants converted glucose to itaconic acid less efficiently. In *pfk*A transformants increased levels of succinic and fumaric acids were detected. With *S. rimosus* a positive effect of *pfk*A and *aox* gene on oxytetracycline production was observed. Characterisation of the *pfk*A transformant showed better specific productivity in respect to the amount of nucleic acid phosphate, RNA, protein and pigment in comparison to the parental strain, while in *aox S. rimosus* transformant significantly increased productivity in respect to pigment production, protein and RNA was observed. The results clearly showed that by enhancing primary metabolic pathways that increase anaplerosis and elevate the pool of intracellular precursors the productivity in both bacterial and fungal cell systems could be increased. Improvement of biotechnological processes by modifying primary metabolism has been generally ignored so far and the results of the ANTICO project should promote a new approach in modification of the commercial micro-organisms.

Acknowledgements This work was financed within the framework of the Cell Factory Programme of the Commission of the European Communities. Quality of Life and Management of Living Resources. ANTICO: QLK3-CT-2002-02038. *Aspergillus niger* genes isolation was done by the groups of Professors Matic Legisa (National Institute of Chemistry, Slovenia) and Anton Glieder (Technical University of Gratz, Austria).

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Preparation of clavulanate salt using a tertiary octylamine as an intermediate

D. Battaglia Hirata[1], J. H. Hortolan Luiz de Oliveira[1], A. G. Ferreira[2], K. Veloso Leão[2], C.P.Sousa[3], M. Barboza[*,1], and C. Osamu Hokka[1]

[1] Universidade Federal de São Carlos, Department of Chemical Engineering, 13565-905, São Carlos-SP, Brazil
[2] Universidade Federal de São Carlos, Department of Chemical, 13565-905, São Carlos-SP, Brazil
[3] Universidade Federal de São Carlos, Department of Microbiology and Patology, 13565-905, São Carlos-SP, Brazil

[*]Corresponding author: e-mail: marlei@power.ufscar.br

This paper describes a study of the preparation of an organic ammonium salt of clavulanic acid by a precipitation reaction. The choice of clavulanic acid was based on its significance for the pharmaceutical industry and on the fact that the precipitation of this organic compound has been little studied and is, unlike inorganic compounds, complex. The objective was to study the influence of the initial concentration of clavulanic acid on the precipitation reaction with a tertiary octylamine. The extraction of clavulanic acid was performed at temperatures less than 20 °C and, preferably, near 15 °C in the water-immiscible organic solvents ethyl acetate. The drying step was performed with a suitable desiccant. Thus, an insoluble salt with clavulanic acid was formed. The precipitate obtained was crystalline, stable and was analyzed by 1H NMR.

Keywords clavulanic acid; solvent extraction; precipitation; t-octylamine

1. Introduction

Clavulanic acid (CA) is a commercially and clinically important β-lactamase inhibitor utilized in conjunction with amoxicillin to overcome the resistance developed by several pathogenic bacteria to this broad-spectrum penicillin [1]. However, CA is a hygroscopic oil and it is not used in this form for the preparation of pharmaceutical compounds. Potassium clavulanate is more stable than the free acid or other salts, such as sodium, calcium and magnesium clavulanate, and is therefore most frequently used in commercial preparations.

CA is produced by fermentation and purified from the fermentation medium in several steps. The steps may involve a variety of standard techniques such as ion-exchange chromatography and solvent extraction of an aqueous solution of impure clavulanic acid [2-4]. Quite often such methods result in a product at low concentration in aqueous solution, which needs to be converted to a clavulanate salt for subsequent freeze-drying or solvent evaporation. These procedures involve raised costs for industry and higher degradation rate as CA in aqueous solutions distant from neutral pH and at temperatures above 30°C [5, 6]. CA conversion to esters or to amine salts that are stables intermediates can be used to enable a CA precipitation from dilute solutions. Nevertheless, some amines give rise to amine salts of clavulanic acid which are either hygroscopic or toxic or both [7]. Thus the choice of an amine, to produce an amine salt of clavulanic acid,is very important.

Published data on the formation of amine salts of clavulanic acid with primary, secondary or tertiary amines is seen only in patens. CA precipitation with a tertiary octylamine allows the formationof a stable salt. This octylammonium salt of clavulanic acid can later be converted to potassium clavulanate by reaction with potassium 2-ethyl hexanoate. This procedure improves the purity of the final product [8].

In the work reported here, the influence of the initial CA concentration on the precipitation reaction with the tertiary octylamine were analyzed , and the yield of the reaction was estimated.

2. Material and methods

Clavulanic acid was obtained from the pharmaceutical product Clavulin® produced by Smithkline Beecham Laboratory.

An aqueous solution of clavulanic acid was acidified with H2SO4 to pH 2.0 and shaken with ethyl acetate (Sigma), which was chosen since water is much less soluble in this solvent than in n-butanol [9].The ethyl acetate was separated and the remaining aqueous solution was re-extracted with ethyl acetate two more times. The organic fractions were combined, dried with a sutable desiccant and concentrated in a rotatory evaporator until a suitable concentration was reached (Table 1).

For the precipitation experiments, the concentrated solution of clavulanic acid in ethyl acetate was transferred to a jacketed glass reactor and maintained at 15°C with a thermostatic bath under continuous stirring (at 250 rpm) with a propeller stirrer connected to a velocity controller. A tertiary octylamine was added dropwise, (1 drop in 90 seconds) and the solution stirred for one hour. The precipitated product was filtered and washed with a little ethyl acetate. The crystals obtained were dried for 24h at room temperature and weighed.

Table 1 Conditions for precipitation experiments combined volume

Experiments	AC Initial Mass (mg)	Initial Volume of Aqueous Solution (mL)	Volume combined of Organics Phases (mL)	AC Conc. in Organic Solvent (mg/mL)	Volume of Amine added (mL)
1A	250	100	150	6,6	0.10
2A	375	100	150	15,0	0.15
3A	500	100	150	23,4	0.20

The yield of crystals (Y), was calculated by Equation 1.

$$Y\% = \frac{m_{exp}}{m_t} \times 100 \quad (1)$$

Here, m_{exp} is a mass obtained through the experiments, and m_t is the theoretical mass for 100% conversion.

2.1 Analytical methods

Clavulanic acid concentration was determined by high performance liquid chromatography (HPLC), as described by Foulstone and Reading [10], through the imidazole reaction. The HPLC system was equipped with a photodiode array detector (Waters 996 PDA) and a C18 -μBondapak analytical column (3.9 x 300 mm). The HPLC equipment was operated at 28°C, with a flow rate of 2.5 mL/min and standard solutions were prepared from the pharmaceutical product Clavulin®.

The thin layer chromatography (TLC) analyses were performed with plastic-backed Silica-gel TLC sheets, eluted with various mixtures of solvents. Plates were visualized by ultraviolet light in 254 nm and by spraying phosphomolybdic acid.

The NMR data were recorded on a Bruker DRX400 9.4 T instrument, operating at 400.35 MHz for 1H and 100.10 MHz for 13C channels, respectively. All NMR data were obtained at 25ºC using TMS as internal reference and D2O as solvent.

3. Results and discussion

The product obtained in all the experiments was a crystalline solid, pale yellow and very stable (not hygroscopic). It can be observed in Table 2 that the yields (Y %) increasing with the decrease of the initial concentration of clavulanic acid.

Table 2 Results obtained from precipitation reaction.

Experiment -	Experimental CA Mass (mg)	Theoretical CA Mass (mg)	Y %
1A	173.51	184.30	94.15
2A	202.00	276.44	73.07
3A	257.80	358.69	69.94

Figure 1 shows the precipitation reaction of CA with the t-octylamine.

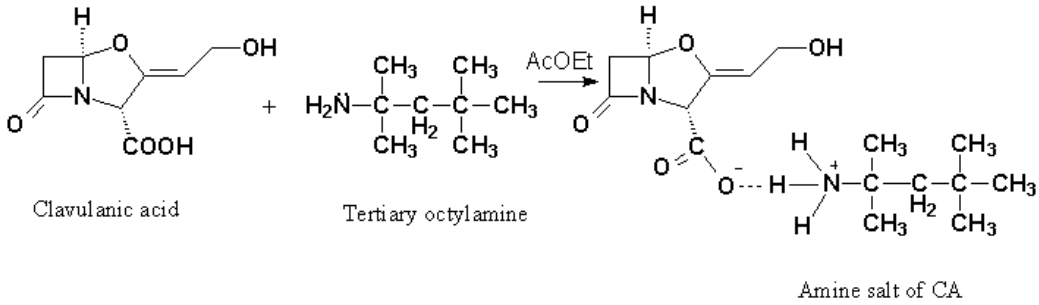


Figure 1 – Precipitation reaction of CA with the t-octylamine

The best yield (Y%=94.15) was obtained in the experiment with the lowest initial concentration of clavulanic acid in ethyl acetate (6.6 mg/mL). This proved the great influence of initial concentration on the precipitation reaction and demonstrated that it is possible to precipitate, this salt of CA from very dilute CA solutions, which is an advantage when the initial extraction is an advantageous when the AC initial solution is from fermentation medium, for example. A fermentation broth with a high initial concentration of CA is not simple to obtain and many articles have been published with attempts to improve the production of clavulanic acid during the fermentation process. Generally, a concentration step is required, to increase the concentration of clavulanic acid, before the precipitation reaction with other amines, esters or potassium 2-ethyl hexanoate.

Precipitation of potassium clavulanate as the final step in the purification of CA is very advantageous, because in this process CA degradation does not occur, in contrast to what happens when solvent or water evaporation is carried out. Besides, it economizes the energy cost to the industry. However the reaction requires very specific conditions, since if not controlled an oil is formed which cannot be used in a pharmaceutical product and results in final loss of product [11].

CA precipitation with a tertiary octylamine has the same advantages as the potassium 2-ethyl hexanoate reaction, allowing stable intermediate salt formation (octylammonium clavulanato), it can also be obtained from very dilute aqueous solutions of clavulanic acid. The reaction conditions are thus not so limited as those with potassium 2-ethyl hexanoate. Also, it is possible to precipitate this salt of clavulanic acid from ethyl acetate, without using any co-solvent. This is an important advantage as the solvent recovery process is much simplified, improving the cost of this process [7].

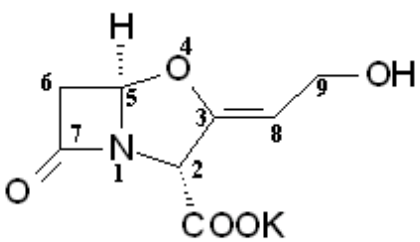
t-Octylamine has advantages over the other amines usually described in patents. Many salts, derived from a wide range of primary, secondary and tertiary amines give rise to salts of clavulanic acid, that are hygroscopic or toxic and it is hoard to isolate the CA from the salts formed [7,8]. Analyzing the NMR-^{1}H spectra for all experiments (Figures 2, 3 and 4) it can be see that practically no substance other than the amine salt of clavulanic acid is detected. Therefore, this intermediate salt may be used for the preparation of highly pure non-toxic clavulanic acid and its pharmaceutically acceptable salts.

Careful analysis of NMR-^{1}H indicate the presence in δ 5,74, 4,96, 4,92, 4,19, 3,58, 3,14 of typical clavulanate hydrogen, as well as the signals regarding amine hydrogens (t-octhylamine), such as methyl groups in δ1,46 and 1,05 and the methylenic group in δ1,69. The chemical shifts of hydrogens added to the signals integration observed in the NMR-^{1}H spectrum indicated the hydrogens exact proportion in the precipitated molecule.
It is also observed the NMR-^{13}C and two-dimensional HSQC, HMBC and COSY spectra which confirmed the proposed structure.

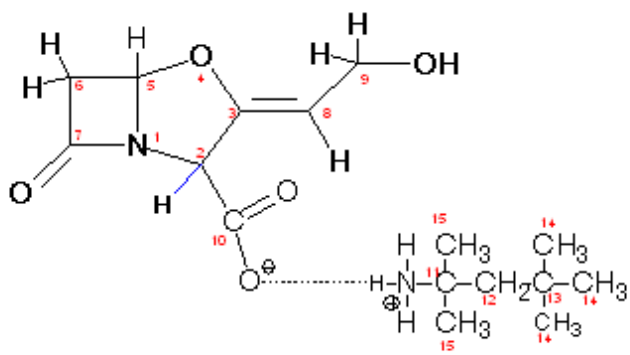
The following table displays the hydrogen and carbon chemical shifts of the potassium clavulanate and the salt of amine clavulanate, it is observed great similarity among the clavulanate moity.

Table 3 Hydrogen chemical shifts (ppm) and J (Hz) from NMR spectrum for clavulanic acid and octylammonium salt of CA(clav/octyl)

Position	$\delta^{1}H$ (*m*, *J* Hz) ClavK	$\delta^{13}C$ Clav K	$\delta^{1}H$ (Clav/octyl))	$\delta^{13}C$ (Clav/octyl)
1 (N)	-	**-**	-	**-**
2 (CH)	4,78 *(dd; 1,18; 1,03)*	**65,1**	4,96	**66,0**
3 (Csp^2)	-	**157,0**	-	**158,2**
4 (O)	-	**-**	-	**-**
5 (CH)	5,67 *(dd; 0,63; 2,13)*	**88,7**	5,74	**90,1**
6 (CH_2)	3,46 *(dd; 2,8; 13,7)* 2,95 *(dd; 0,67; 15,8)*	**46,6**	3,58; 3,14	**48,1**
7 (CO)	-	**178,2**	-	**182,3**
8 (CH)	4,89	**99,0**	4,92	**100,4**
9 (CH_2)	4,18 *(ddd; 0,85; 7,42;12,2)* 4,12 (*ddd; 1,1; 6,99; 12,3)*	**57,7**	4,19	**58,7**
10 (COOH)	-	**174,8**	-	**177,1**
11 (Csp^3)			-	**59,2**
12 (CH_2)			1,69	**54,8**
13 (Csp^3)			-	**-**
14 ($3CH_3$)			1,05	**33,2**
15 ($2CH_3$)			1,46	**29,3**



Clav K



Clav/octyl

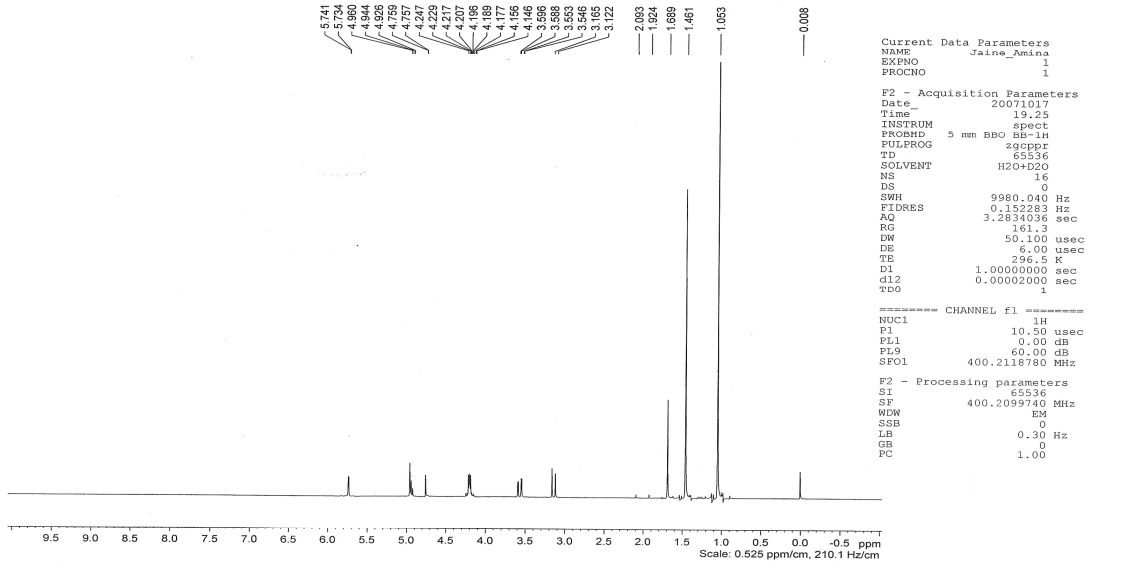


Figure 2 – NMR-^{1}H spectrum of precipitated salt of clavulanic acid with t-octylamine from assay 1A.

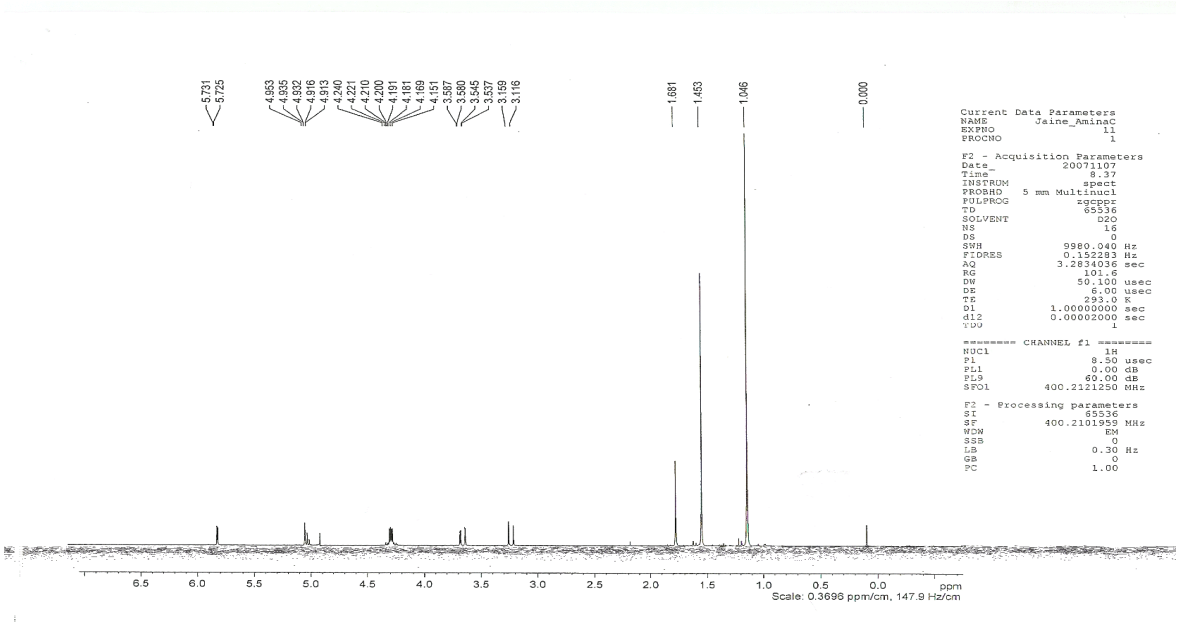


Figure 3 – NMR-^{1}H spectrum of precipitated salt of clavulanic acid with t-octylamine from assay 2A.

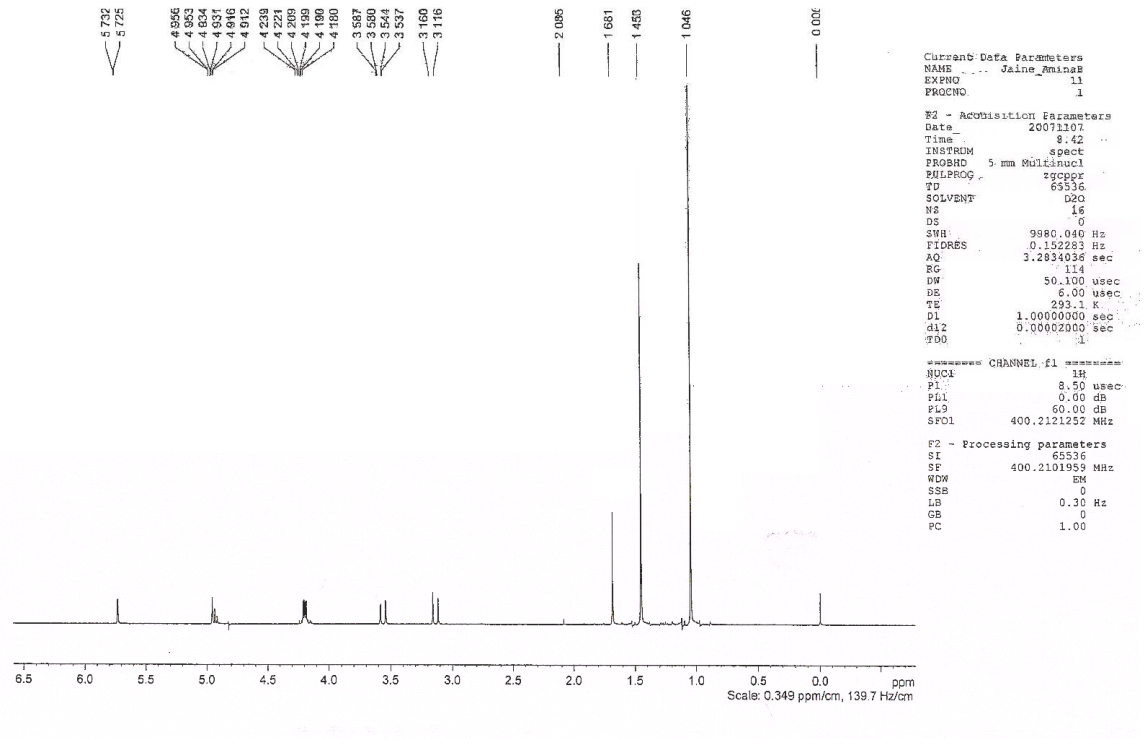


Figure 4 – NMR-^{1}H spectrum of precipitated salt of clavulanic acid with t-octylamine from assay 3A.

4. Conclusions

From the reported results, it is possible to conclude:

- The use of a tertiary octylamine to precipitate clavulanate has the added the salt of clavulanic acid from very dilute solutions.
- A higher concentration of CA caused a decrease in the yield of precipitation with tertiary octylamine.
- Precipitation is a very promising process, since no solvent evaporation techniques are necessary, avoiding degradation of CA and it is possible to obtain a product of high purity.
- Addition of a co-solvent is unnecessary, simplifying the solvent recovery process, wich is therefore less hostile to the environment.

Acknowledgements: The authors gratefully acknowledge the financial support of FAPESP (Grants 04/16056-6, 05/56982-0 and 05/55079-4)

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Recovery of clavulanic acid from an aqueous two-phase system by ion-exchange resin

C. Sacardo da Silva[1], V. O.Barreto[1], E.Bavarotti[1], G.Youssef Rodriguez[1], C. Osamu Hokka[1], and M. Barboza[1]

[1] Department of Chemical Engineering, UNIVERSIDADE FEDERAL DE SÃO CARLOS, São Carlos 13565-905, SP, Brazil

A study was made of the process of clavulanic acid extraction from filtered fermentation broth by an aqueous two-phase system and recovery of the clavulanic acid by ion-exchange resin. The extraction experiments were carried out using a polyethylene glycol (PEG)/potassium phosphate aqueous system at pH of 6.0 and a PEG molecular weight of 600 or 6000. After the extraction, the antibiotic-rich PEG phase (light) solution was re-extracted with ion-exchange resin (Amberlite IRA-400). This paper discusses the conditions, procedure and results obtained in the process of clavulanic acid extraction and purification. Each step of the process proposed here was evaluated from the standpoint of yield (Y), partition coefficient (Kp), losses by decomposition of clavulanic acid (D). The results indicated a recovery of clavulanic acid varying from 50 to 80, Kp varying from 2 to 5, and D varying from 18 to 76%. In conclusion, clavulanic acid was extracted from fermentation broth into the PEG phase of a two-phase aqueous system, from which it was re-extracted by ion-exchange resin in a column. The results confirm the viability of this two-stage metohod

Keywords clavulanic acid, back-extraction

1. Introduction

The first step of isolation and purification of the clavulânico acid (CA) in the broth is the primary clarification process by filtration or centrifugation. Owing to its naturaly unstable free form, the CA molecules are isolated in the form of its lithium, sodium or potassium salts [1-5]. Its chemical instability causes high rates of degradation at extremes conditions of pH (less than 4.5 and more than 7.5) that lead to reductions in the yield during purification [6].

However, in view of the growing therapeutic and commercial importance of CA, much research is aimed at finding new and modern techniques for its purification. There are in the literature many studies that involve the utilization of the polymeric ion-exchange resin Amberlite IRA-400 for the purification of CA. This resin is able to provide a considerable degree of purification of CA, and all its kinetic, thermodynamic and mass transference properties had been studied [7-9], as have the fixed-bed and continuous separation processes [10]. The separation of CA by aqueous two phase system, ATPS, is reported in just one article, by Videira & Aires-Barros (1994) [11]. In this study a PEG/phosphate system, at pH 7 and 8, was used and clavulanic acid solution was obtained from the pharmaceutical industry. The authors observed a high affinity for the PEG-rich phase, with high partition coefficients and recoveries. Up to the present, experiments involving fermented medium have not been reported in the literature.

The general objective of this study was to develop a protocol that permits the extraction of CA from fermentated broth with ATPS and its re-extraction with ion-exchange resins as an alternative to the organic solvent extraction and purification processes.

2. Materials and Methods

2.1 Materials

a) Extraction step:

Clavulanic Acid was produced by *Streptomyces clavuligerus* in fermentation medium which contained glycerol as carbon source and Samprosoy 90 NB® as nitrogen source [12]. The broth was first micro and ultrafiltration: For the extraction with ATPS, polyethylene glycol (PEG) of molecular 600 or 6000 and phosphate buffer were utilized and the CA was extracted from the PEG-6000 or PEG-600 phases (top phases).

b) Back-extraction step:

The recovery experiments were run in fixed bed columns, models SE-10-50 (10mm) and XK-16 (15 mm) from Pharmacia. The fixed bed was 265 mm of and the ion exchange resin Amberlite IRA 400 Cl, from Rohm and Haas. The CA was desorbed from the resin with 5% (w/w) NaCl solution. The mobile medium was the light phase from the ATPS stage which contained PEG + CA as main components. The ATPS preparation is described in the in the methods below.

2.2 Methods

a) Resin treatment:

The resin is preteated by by passing 3 fixed-bed volumes of sodium chloride (NaCl 5% w/v) solution through the column followed by Milli-Q water for 30 minutes. This procedure ought to be repeated after the resin is used and it must be stocked in aqueous ethanol solution (20% w/w).

b) Fermentation broth treatment:

For the ATPS steps, the pH was adjusted to 6.2. The fermentation broth was previously filtered through by micro and ultrafiltration membranes, by the procedure giben in according to Silva *et al.* (2007) [13].

c) Preparation of the ATPS

The ratio (w/w) of PEG (15%) to phosphate (17%) was found on the phase diagram from Zaslavsky (1995) [14] and used to calculate the weights needed to prepare the ATPS, besides the weight of ultrafiltered broth.

d) Determination of CA concentration:

The concentrations of CA in the fermentation broth solutions were found by high-performance liquid chromatography (HPLC), as described by Foulstone and Reading (1982) [15] with imidazole reaction. An HPLC device with a Photodiode Array detector (Waters 996 PDA) was utilized with a reversed-phase column (C18 µ-Bondapak 3.9x300 mm).

e) Determination of PEG concentration:

The PEG concentration was measured by UV spectrophotometer by the B. Skoog (1979) [16] method: 1.0mL sample is shaken with 5 mL diluted perechloric acid (0.5M). to 4 mL of this mixture, 1 mL of a 5% barium chloride solution and 0.5ml of a 0.1M iodine solution (Merck, Titrisol® 9910) were added. The absorbance at 535nm was then recorded in the spectrophotometer after 15min. Calibration solutions, containing PEG but no CA, were treated in the same way as the unknown samples.

3. Experimental Procedure

First separation step

The fermentation broth was firstly filtered through micro and ultra filtration membranes. The sketch of the equipment is showed at Figure 1.

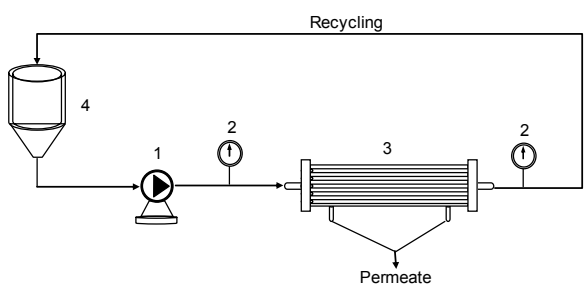

Fig. 1 Experimental set up of the filtering equipment used in the experiments.1- Peristaltic pump; 2-Manometer; 3-Membrane; 4- Broth reservoir.

The microfiltration and ultrafiltration operating conditions such as transmembrane pressure, flow and temperature, were those established by Silva *et al.* 2007 [13].

ATPS extraction step

The extractions were made in aqueous two-phase systems that contained PEG-6000 or PEG-600 solution in Milli-Q water (50% w/w) and a solution of mono (16.97% w/w) +dibasic (13.03% w/w) phosphate in Milli-Q

water complemented with ultrafiltered broth, to reproduce the weight percentage of each substance found at the Diagram of Phases. At the end of the phase's separation, it was possible to calculate the partition coefficient, defined in Equation 1.

$$K_p = \frac{C_{CAtop}}{C_{CAbottom}} \quad (1)$$

Back-extraction of CA from the light phase

The re-extraction of CA by ion exchange adsorption with the resin Amberlite IRA-400 was evaluated. A sketch of the equipment is shown in Figure 2.

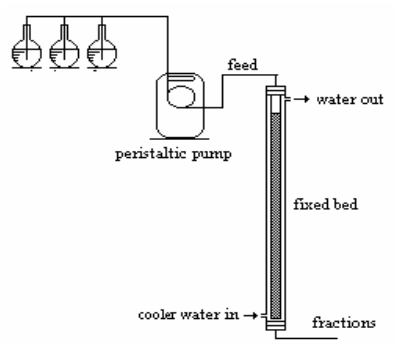

Fig. 2. Experimental setup of adsorption experiments

A thermostatic bath was used to maintain the columns at 20ºC. The experiments to obtain the breakthrough curves were carried out by feeding the column continuously with the pretreated medium. After the saturation of the column, it was washed with Milli-Q water to remove the excess PEG. After that the CA was eluted with NaCl solutions. Small samples were periodically (10mL/min) collected from the column effluent and the CA and PEG concentrations were determined by the methods already described. The procedure was repeated with the column temperature at 10ºC and 30ºC.

At the end of each experiment, the results were analysed and compared to decide which PEG and temperature were best for the extraction and re-extraction procedures. The aim was to combine the highest concentration (CF) and yield (Y) factors with the lowest losses by decomposition of CA (D), as calculated by etquation (2) to (5).

$$CF_{(CA)} = C_{(pCA)} / C_{0CA} \quad (2)$$

$$CF_{(PEG)} = C_{(pPEG)} / C_{0PEG} \quad (3)$$

$$Y = \sum C_{(CA)} / C_0 \quad (4)$$

$$D = 1 - Y \quad (5)$$

In equations 2 and 3, C_{0CA} and C_{0PEG} are the initial CA and PEG concentrations, respectively, and $C_{(pCA)}$ and $C_{(pPEG)}$ are respectively their maximum concentrations at the outlet from the column.

The PEG concentrations were determined by equation 6, where y is the spectrophotometer reading and F is the dilution factor.

$$C_{(PEG)} = F.y \quad (6)$$

4. Results and Discussion

The results were analyzed by calculating Kp CF, D and Y with the equations 1 to 5, from the results obtained with the HPLC and the spectrophotometer. The values of Kp, CF, D and Y in each experiment are shown in Table 1, with the respective molecular weight of PEG, temperature, column flow rate and resin volume.

Table 1. Experimental data from the re-extraction of CA with the resin Amberlite IRA400, in fixed-bed column, pH 6,2

PEG	T (°C)	Flow (mL/min)	V_{resin} (cm^3)	$CF_{(CA)}$ (-)	$CF_{(PEG)}$ (-)	D (%)	Y (%)	Kp (-)
6000	20	4.0	16.5	0.46	1.3	46.42	53.58	2.31
600	10	6.0	46.5	0.66	-	18.38	81.62	5.10
600	20	6.0	46.5	0.72	4.36	23.85	76.15	5.10
600	30	6.0	46.5	0.59	-	23.68	76.32	3.14

According to Table 1, the extraction of clavulanic acid from the fermentation medium by the PEG-phosphate aqueous two-phase system is a very promising process as it was possible to obtain partition coefficients (K) higher than unity. Table 1 also shows that PEG-6000 provides a lower CF and higher losses, during extraction of CA, than PEG-600 at the same temperature of 20°C. Therefore, PEG-600 was used to study the influence of temperature. The CA and PEG concentration profiles during the back-extraction runs with Amberlite IRA -400 are shown in Figures 3 and 4.

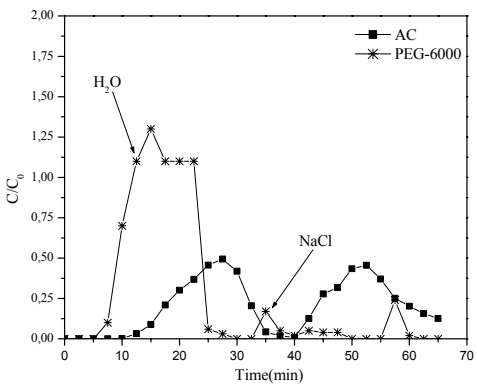

Fig. 3. Back-extraction of CA from PEG-6000 phase, 20°C.

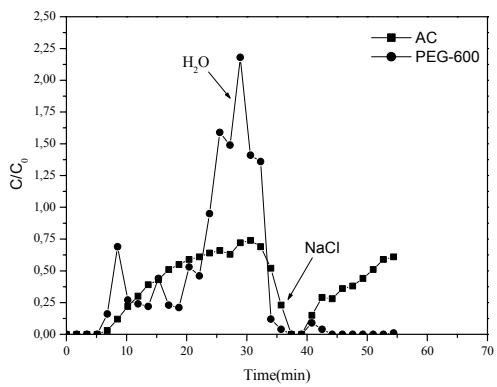

Fig. 4. Back-extraction of CA from PEG-600 phase, 20°C.

The saturation of the column takes from 0 to 5 minutes, after which first CA not adsorbed by the column emerges. The passage of water drove the excess PEG from the column, so that it was totally eliminated, except at the temperature of 10ºCsince the increase in its viscosity at the lower temperature, himdered its passage through the column. The CA saturation results are shown in Figure 5.

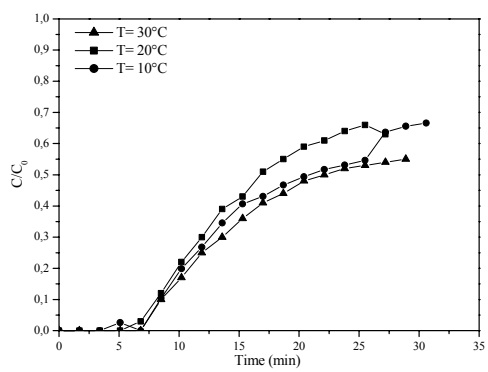

Fig. 5. Saturation curves of CA in the resin Amberlite IRA-400.

A reason that CF does not reach the maximum value of 1 (100%) is that there are sites on the resin Amberlite IRA-400 that promote CA degradation.

5. Conclusion

The results revealed that it was possible to re-extract CA from PEG phase.

The best CF was obtained with the PEG-600, at a temperature of 20°C, while the smallest D was also achieved with PEG-600, but at a temperature of 10ºC.

Moreover, the absence of detectable PEG during the elution of CA proves that sodium chloride (5% w/w) is an efficient eluent for this resin.

Abbreviations

CA: Clavulanic Acid;
$C_{(pCA)}$: Peak concentration of CA (mg/L);
$C_{(pPEG)}$: Peak concentration of PEG (mg/L);
C_0: Initial concentration (mg/L);
$C_{(CA)}$: CA concentration (mg/L);
DF: Degradation factor (%);
$CF_{(CA)}$: Concentration Factor of AC;
$CF_{(PEG)}$: Concentration Factor of PEG;
F: Dilution factor;
PEG: Polyethylene glycol;
y: HPLC and spectrophotometer measures.

AcknowledgementsThe authors gratefully acknowledge the financial support of FAPESP (Grants 04/16056-6, 05/56982-0 and 05/55079-4) and scholarship granted to V. Barreto, G.Youssef Rodriguez (Laboratory Science Training) and CNPq for the doctored scholarship granted to C.S.Silva.

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Study of Fouling Index in Tangential Filtration Applied for Separation of Clavulanic Acid from Fermentation Broths

C. S. da Silva, G.Y Rodriguez, C. O. Hokka, M. Barboza[1]

[1] Department of Chemical Engineering. Universidade Federal de São Carlos

Clavulanic acid is a potent inhibitor of beta-lactamases produced by *Streptomyces clavuligerus* cultures. Among the various types of separation and purification processes in use, microfiltration and ultrafiltration are outstanding for providing purer broths that will result in higher yields in subsequent liquid-liquid extraction and/or chromatography stages. The purpose of this study was to analyze the behavior of the permeate flux in tangential filtration, carried out with MF (0.2 μm cut-off) and UF (50 kDa and 3 kDa cut-off) membranes. Various transmembrane pressures (P_{TM}) were applied. In the MF experiments, it was evident that higher feed velocities and lower P_{TM} were preferable. Regarding UF, it was found that raising P_{TM} did not lead to a proportional rise in flux.

Keywords Clavulanic acid, fouling index.

1. Introduction

Separation membranes are applied to a wide range of tasks and these can be classified broadly as microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF). MF and UF are widely used as a first step in the clarification of fluids or recovery of solutes. In recent years, research workers have explored the use of membranes to separate antibiotics [7, 13-14, 18], among them the β-lactam antibiotic clavulanic acid (CA) [10-12]. CA is produced industrially by microbial fermentation and is isolated and purified from the culture medium in several steps. The medium is first clarified by filtration or centrifugation and this is followed by adsorption or liquid–liquid extraction with organic solvents, normally butanol [2]. CA is degraded rapidly during processing and thus falls to low concentrations in the purification step. The rate of decomposition is higher both at acid pH, below 5.0, and in the alkaline range, above 7.0, and it is markedly accelerated when the temperature is raised. Hence, the separation and purification of CA tends to show very low yields, compared to other β-lactam compounds [9]. As a consequence, the time taken for separation is a critical parameter for productivity. MF and UF appear to be suitable alternative methods for the separation of biomass and proteins from the CA fermentation broth, in view of the flexibility of such techniques [4]. However, the steady decline in the flux of permeate during filtering has negative financial consequences and demands a diafiltration stage, which in dilutes the broth feather. This drop in the flow-rate through the membrane in MF and UF is associated with polarization and accumulation of a layer of gel and fouling of the membrane, which can sometimes lead to such low permeate fluxes that the process is inviable. This process of membrane fouling is influenced by several operational variables, including the upstream cross-flow rate, the applied pressure and temperature, the size of the particles in suspension, conditions in the medium and characteristics of the membrane [1, 8].

In view of the potential advantages of applying membrane techniques to the separation and purification of clavulanic acid, the present study was elaborated in order to assess the influence of temperature, hydrostatic pressure drop across the membrane, feed rate and rheological properties of the broth on the permeate flux and on the formation of the gel layer that leads to membrane fouling, in commercial membranes used for MF and UF.

2. Materials and Methods

2.1. Production of clavulanic acid broth

All tests were carried out on broths fermented with *Streptomyces clavuligerus* ATCC 27064. The culture medium consisted essentially of a solution of glycerol and the soy protein isolate Supro 783 (The Solae Company), supplemented with ornithine, mineral salts, $(NH_4)_2\ SO_4$ and KH_2PO_4 [15]. In one of the microfiltration tests (designated M1), a similar culture medium was used, but with the ornithine replaced by soy oil and the Supro 783 by Prosan soy flour (The Solae Company), as described by Ortiz [16]. At the end of the fermentation, the broth pH was adjusted to 6.2 with 18N phosphoric acid, cooled to either 11ºC or 20ºC and passed through tubular microfiltration membranes. The permeates from this step were then used in the study of ultrafiltration.

2.2. Clavulanic acid determination

The concentration of CA was measured by the spectrophotometric method published by Bird [17].

2.3. Membranes

The following hollow-fibre membranes were used for MF and UF: a)MF: Polysulfone membrane with 0.2μm diameter pores b) UF: Polysulfone membranes of pore sizes 3kDa and 50 kDa, (both supplied by Amersham Biosciences).

2.4. Rheology tests

A Brookfield dial-reading DV-III viscometer (Brookfield Engineering Laboratories Inc.) was used to measure the rheological properties of the fluids.

2.5. Operation of the filtration unit

The experimental set-up is shown in Figure 1. The tangential filtration system was composed of a feed reservoir initially filled with broth. The test fluids were made to pass through the filtration module with the aid of an Ismatec peristaltic pump. The pressure drop across the membrane was regulated by a Mipel needle valve V1. The concentrate was recycled back to the reservoir and the permeate was collected in a test-tube, its volume (Vp) being recorded throughout the test.

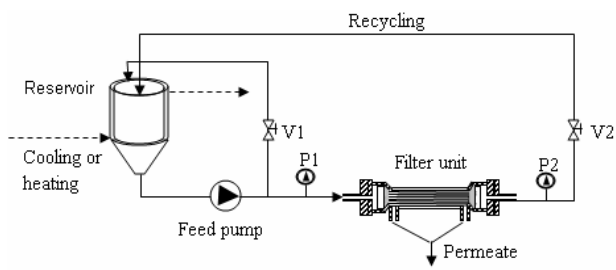

Fig. 1. Experimental set-up for tangential filtration. P1, P2: Bourdon pressure gauges. V1, V2: needle valves to control mean pressure drop.

In all, 6 MF and 6 UF experiments were carried out with the apparatus shown in Figure 1. The ultrafiltration experiments were all performed at 11ºC with a constant cross flow rate of 21.6 $L.h^{-1}$, but varied in pressure drop across the membrane (P_{TM}). In three UF experiments, U1-U3, a membrane of pore size 50kDa was tested. In another three experiments, U4-U6, a 3kDa membrane was used.

2.6. Definition and measurement of pseudo steady-state permeate flux (J_{ps}) and fouling index (I)

The theoretical equations used to represent conventional dead-end filtration are slightly different from those for tangential filtration. In practice, however, the difference is more apparent than real [12] and the flux through the membrane, J, can be related in the usual way, to the pressure drop in the system (ΔP), or rather the transmembrane pressure drop (P_{TM}) in tangential filtration, and the resistance to flow through the membrane:

$$J = \frac{1}{A_m}\frac{dV_P}{dt} = \frac{P_{TM}}{\mu_P(R_m + R_c)} \quad (1)$$

where μ_p is the viscosity of the permeate, R_m and R_c are the flow resistances due to the membrane and the cake (gel plus solids) accumulating on its upstream surface, respectively, and P_{TM} is the average pressure drop across the membrane, given by eq.2.

$$P_{TM} = \frac{P1 + P2}{2} - P_p \quad (2)$$

where P_p is the hydrostatic pressure in the downstream permeate. R_m is constant for a given filter and broth. R_c is a function of the specific resistance of the accumulating cake,α, the filtering surface area, A, the collected

volume of permeate collected, V_p, and the bulk concentration of particles per unit volume of permeate, C_b, as shown in eq.3. In most cases it is impossible to determine C_b and α, accurately. For convenience, the fouling index (I) is defined as αC_b. Substituting R_c from Eq 3 into Eq 1 gives Eq. 4

$$R_c = \frac{V_P}{A} \times \alpha C_b = \frac{V_P}{A} \times I \tag{3}$$

$$J = \frac{P_{TM}}{\mu_p \left(R_m + \frac{V_P}{A} I \right)} \tag{4}$$

Rewriting V_p/A as a product of flux, J and time t, $J.t$, and rearranging gives the following equation:

$$P_{TM} = J\mu_p R_m + J^2 \mu_p I t \tag{5}$$

The fouling index was calculated at time (t) according to the following equation 6. The cake resistance (R_c) can be assumed to reach a maximum value, implying that the permeate flux (J_p) falls to a minimum (J_{ps}) at the pseudo steady state. The less the influence of the resistances of the membrane and cake resistences, the higher will be the value of J_{ps} and thus the greater the productivity. To determine the fouling index (I), it is necessary to monitor the increasing permeate volume (V_p) over time and analyze these data with equations 1 to 6.

$$I = \frac{P_{TM} - J\mu_p R_m}{J^2 \mu_p t} \tag{6}$$

2.7 Reological properties of suspensions

For fluids, such a suspension of *Streptomyces clavuligerus* in broth, which follow the rheological power law, the shear stress(τ) is related to the shear rate (γ) as follows:

$$\tau = K\gamma^n \tag{7}$$

In this expression n is the flow index and K, the consistency index. The apparent viscosity (η_a)of the fluid is represented by Eq 8. For Newtonian fluids the flow index is 1 and the viscosity (μ) is equal to consistence index.

$$\eta_a = K(\gamma)^{(n-1)} \tag{8}$$

3. Results and Discussion

3.1. Membrane characterization

For the membranes used in this study, the values of R_m are listed in Table 1.

Table 1. Values of resistances of membranes

Membrane of filtration	R_m (m^{-1})
0.2μm	2.14 x 10^{11}
50kDa	3.32 x 10^{12}
3kDa	2.14 x 10^{13}

3.2. Experiments with microfiltration membrane

The initial operating conditions and experimental results obtained in the microfiltration of the fermented broth are given in Table 2 and Figures 2, 3, 4 and 5. The effect of P_{TM} on J_{ps} can be observed in these data, by

comparing the results in experiments M3 and M4, since both were carried out at 20ºC and a feed flow-rate of 21.6 $L.h^{-1}$. The value of J_{ps} did not change significantly with the rise in the pressure drop from 98 to 122 kPa, although the higher P_{TM} would be expected to produce a greater flux of permeate. On the other hand, it should be noted that the most resistant gel was formed in experiment M3. This was due to the initial consistency index (or stiffness) of the broth, K_{init}, being higher in M3 than in M4. K_{init} increases with the cell density and the content of dissolved and suspended solids. Given that the fouling index (I) is directly proportional to the specific cake resistance (α, a function of the constituents of the cake) and the dry mass of cake formed (C_b), which in turn is directly proportional to the total solids content in the feed, it can be seen that I should rise with K_{init}. In the present case, the greater gel resistivity in M3 prevented the permeate flux from being increased by the higher P_{TM}.

Table 2. Operating conditions and results obtained in MF experiments wich CA fermentation broths.

Expt.	Initial and operating conditions				Experimental data obtained		
	T (°C)	P_{TM} (kPa)	$Q^{(a)}$ ($L.h^{-1}$)	K_{init} ($Pa.s^n$)	V_p (L)	$t_{op}^{(b)}$ (h)	J_{ps} $L.h^{-1}.m^{-2}$
M1	11.0	98.0	21.6	4.0	1.1	0.66	3.3
M2	11.0	122.0	43.5	1.6	2.4	0.77	5.6
M3	20.0	122.0	21.6	0.5	2.0	1.18	3.3
M4	20.0	98.0	21.6	0.3	1.8	1.12	3.1
M5	20.0	98.0	43.5	0.1	2.5	0.93	2.7
M6	20.0	39.2	43.5	0.3	1.3	0.93	1.4

***Key*:** Q = feed flow-rate; t_{op} = operating time.

The effect of the feed flow-rate through the filtration unit (Q) should be visible in Figure 4, which refers to experiments M4 and M5. The faster feed rate promotes greater turbulence in the flow, diminishing the resistance to flow as the mass-transfer coefficient for the broth-gel interface is raised. However, the actual value of J_{ps} does not reflect these effects, being practically the same in M4 and M5. On the other hand, the effect of feed rate can be observed in experiments M1 and M2, in which there is a modest difference in P_{TM}, whereas the feed rate in M2 is twice that in M1 (Figure 2). It should be noted that in the experiments at 11ºC, M1 and M2, the observed values of K_{init} were much higher than in the other experiments. The broths used in M1 and M2 were produced in conditions differing markedly from the rest. In the case of M2, the culture of *S. clavuligerus* was stopped within the exponential growth phase, while the other cultures were taken to the stationary phase. The high K_{init} shows the high total concentration of substrates (glycerol and Supro 783) that still had not been converted to biomass, CA and proteins, so that the accumulation of gel, reflected in the resistivity I, was the lowest in all the experiments. This resulted in the highest J_{ps} observed in this study (5.6 $L.h^{-1}m^{-2}$). As mentioned in Materials and Methods, the broth in M1 contained Prosan R® defatted soy flour in place of Supro 783®, was supplemented with soy oil and lacked ornithine. Prosan R has around 20% (w/w) fiber content, while the soy protein isolate Supro 783 has only 2.18% (w/w). The value of K_{init} was much higher in M1 than in M2 and, as observed for experiments M3, M4 and M5, this led to more gel formation (higher I). Thus, the flux (J_{ps}) is limited by the gel resistance (R_c) and the formation of gel depends on the consistency index of the broth. However, the nature of the material composing the broth can lead to a high or low R_c, independently of the index K_{init}

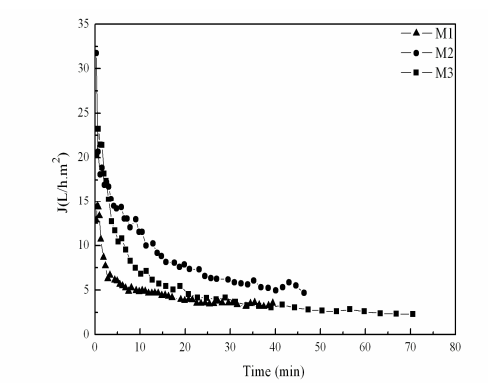

Fig. 2. Permeate flux during experiments M1, M2, M3.

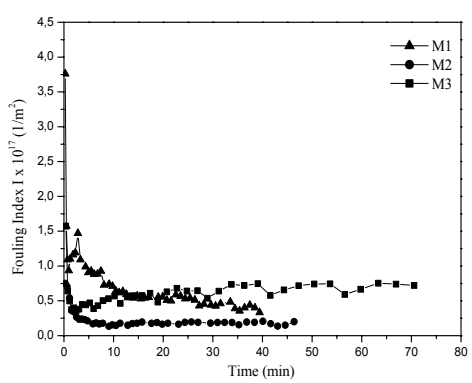

Fig. 3. Fouling index during experiments M1, M2, M3.

Therefore, various characteristics of the broth favor greater or smaller gel formation, which in turn limits the permeate flow. Regarding the membrane resistance (R_m), it can be seen that an increase in pressure-drop from 39 to 98 kPa led to a higher R_m. However, the analysis has to involve other factors, since in experiments M3 and

M4, R_m was much higher at a P_{TM} of 98 kPa than at 122 kPa. The value of K_{init} and the nature of the fluid in the membrane must also be taken into account. Thus, in M2 and M3, higher K_{init} is associated with a higher R_m. Specifically in experiment M2, as already discussed, despite the high value of K_{init}, the small amount of cake formed on the membrane allowed the system to attain its highest permeate flux and caused little damage to the membrane, possibly because the cake in M2 had a low content of colloidal particles. In contrast, the cake formed in experiment M3 was the thickest in all the experiments and thus may have protected the inner zone of the membrane from deformation by alleviating the pressure drop of 122 kPa.

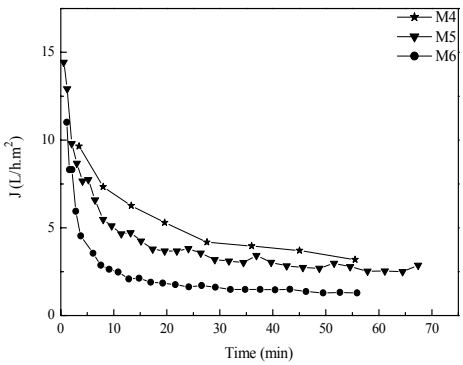

Fig. 4. Permeate flux during experiments M4, M5, M6.

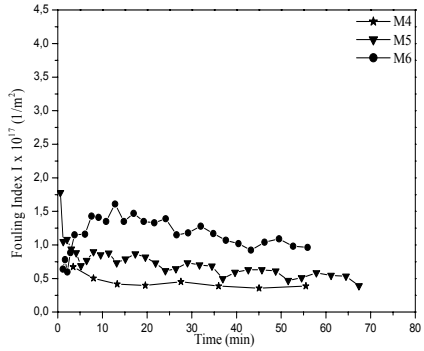

Fig. 5. Fouling index during experiments M4, M5, M6.

Comparison of the results in experiments M5 and M6 (Fig.4 and Fig.5) reinforces earlier conclusions. In M5, K_{init} is considerably lower and P_{TM} much higher that in M6, allowing the filter to attain a faster final permeate flux while accumulating less gel (in exactly the same time). As might be expected, in order to achieve a higher J_{ps} and low I, it is necessary to operate the system at a greater transmembrane pressure, but particular attention must be given to the index K_{init} and other characteristics of the broth, which depend on the age of the culture, as these are deciding factors in the build-up of the gel that ultimately controls the process.

3.3. Experiments with ultrafiltration membrane

The results obtained in UF experiments with the 50 kDa membrane are displayed in Table 3 and Figs 6 and 7. All these tests were run at 11ºC, with a feed flow-rate of 21.6 $L.h^{-1}$. It can be seen that the transmembrane pressure and J_{ps} are related as expected, higher pressure resulting in greater permeate flux. With regard to I (Fig7), all values obtained in these 50kDa UF tests are greater than those observed in MF. This is due to the smaller pores in the UF membrane, which retains a greater quantity of soluble protein than the MF membrane.

Table 3. Operating conditions and results obtained with 50 kDa membrane.

	Initial and operating conditions				Experimental data obtained		
Expt.	T (°C)	P_{TM} (kPa)	Q ($L.h^{-1}$)	μ(Pa.s)	V_p (L)	t_{op} (h)	J_{ps} ($L.h^{-1}.m^{-2}$)
U1	11.0	49.0	21.6	0.2	0.56	1.61	9.9
U2	11.0	73.6	21.6	0.2	0.48	1.49	13.5
U3	11.0	98.0	21.6	0.2	0.40	1.55	14.6

It can be observed in Fig 7 that the fouling index parameter is the same in all tests after the steady state is reached. The results demonstrate the effect of transmembrane pressure on the productivity of system. In the range studied, the fouling index parameter does not cause losses in the permeate flux. In the experiments carried out with the 3kDa membrane, the results followed the expected trend (Table 4 and Fig 8). These tests were also run at 11ºC, with a cross-flow rate of 21.3 $L.h^{-1}$. The initial values of initial I obtained with the 3 kDa membrane, which can be seen in Fig 9, were lower than those with the 50 kDa (Fig. 7) membrane, indicating that a smaller amount of dissolved material such as protein was retained on this membrane and that a thinner cake was probably formed, allowing improved permeate flux. From this result it may be concluded that the permeate solution leaving the MF unit can be made to pass through the 3kDa membrane without great hindrance, as this membrane rejects most of the biomolecules and suffers little deterioration in the process. Although the values of I are lower on the 3kDa membrane, the flux J_{ps} is inferior to that observed with the 50 kDa membrane, at P_{TM} = 73.6 and 98 kPa, due to the resistance of the membrane itself, R_m, which is greater for smaller pores.

Table 4. Operating conditions and results obtained with 3kDA membrane.

Expt.	Initial and operating conditions				Experimental data obtained		
	T (°C)	P_{TM} (kPa)	Q (L.h^{-1})	μ(Pa.s)	V_p (L)	t_{op} (h)	J_{ps} (L.h^{-1}.m^{-2})
U4	11.0	73.6	21.6	0.2	0.23	1.68	11.9
U5	11.0	98.0	21.6	0.2	0.30	1.85	13.3
U6	11.0	122.0	21.6	0.2	0.33	1.95	15.2

Analyzing the behaviour of the fouling index in respect of the pseudo-stead state; it was observed that the UF with 3kDa reached it 55 min, while the UF with 50 kDa did so in 30 minutes. These results support the hypothesis that a smaller amount of dissolved material is slowly deposited in the pores of the membrane where mass transfer phenomenona are more significant. On the other hand, after the steady state of filtration is reached, the values of fouling index are close for both UF units.

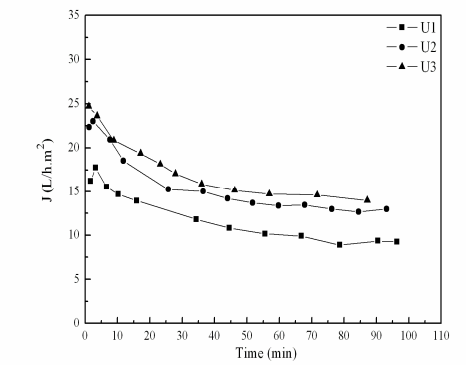

Fig. 6. Permeate flux for 50 kDa membrane. Experiment U1: P_{TM} = 49.0 kPa; U2: P_{TM}= 73.6 kPa; U3: P_{TM} = 98.0 kPa.

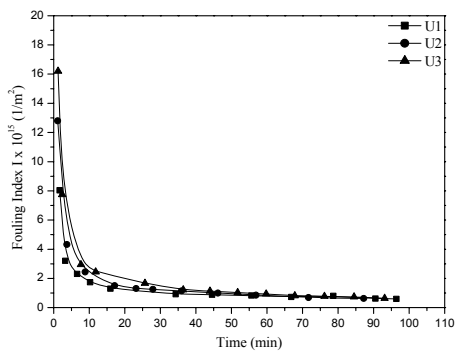

Fig. 7. Fouling index during experiments U1, U2, U3.

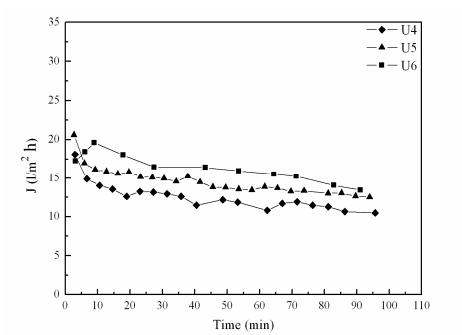

Fig. 8. Flux through 3kDa membrane. Exp. U4: P_{TM} = 73.6 kPa; U5: P_{TM} = 98.0 kPa; U6: P_{TM} = 122.0 kPa.

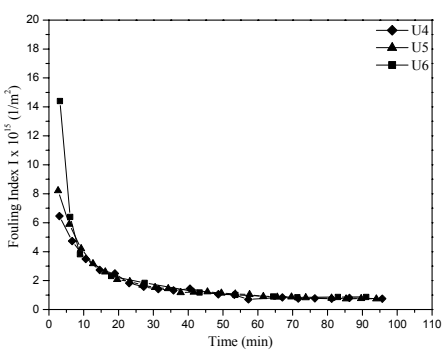

Fig. 9. Fouling index for experiments U4, U5, U6.

The losses of CA during MF and UF were found by measuring concentrations upstream and downstream of the membranes and calculating the mass balance of each process, so as to assess the sources of loss. Loss by degradation was calculated with the aid of the kinetic analysis of CA degradation published by Bersanetti [9]. In none of the MF or UF experiments did such losses exceed 2% of the total CA. However, the total loss during MF was, on average, 20% of the CA, while during UF it was never more than 10%. Thus, the losses are assumed to represent the CA that remained in the valves, inside the filter cartridges and in or on the gel formed on the membrane.

4. Conclusions

It can be concluded that the equation (1), usually applied to dead end filtration, is suitable to predict the permeate flux through the membrane in cross-flow filtration, in batch operation.

It has been shown that the flow resistivity (**I**) of the microfiltration cake is governed by the initial conditions of the fermentation broth to be filtered. The MF permeates proved totally free of colloids. In ultrafiltration (with 3 or 50 kDa membranes), a rise in pressure leads in general to a greater flux of permeate; however, this is not

guaranteed, owing to the increased polarization of the gel layer and greater membrane resistance that accompany a higher P_{TM}. The joint experiment (MF and UF) provided evidence that knowledge of the initial conditions of the broth to be filtered is crucial to achieving an adequate permeate flux. To lengthen the useful life of the membrane, with negligible productivity losses, intermediate values of P_{TM} and high tangential velocities should be used. Finally, the recommended operating conditions emerging from this study are as follows: for MF, a P_{TM} of 98 kPa, temperature of 11ºC and feed flow-rate 21.6 $L.h^{-1}$; for UF, a temperature of 11ºC, feed flow-rate of 21.6 $L.h^{-1}$ and P_{TM} not exceeding 80 kPa.

Acknowledgements The authors gratefully acknowledge the financial support of FAPESP (Grants 04/16056-6, and 05/55079-4) and the CNPq scholarship granted to C.S. Silva (Doctorate).

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