# Biochemistry and Biotechnology

## Research and Development

Biochemistry Research Trends

Sergey D. Varfolomeev
Gennady E. Zaikov
Larisa P. Krylova
Editors

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# BIOCHEMISTRY AND BIOTECHNOLOGY

# RESEARCH AND DEVELOPMENT

SERGEY D. VARFOLOMEEV
GENNADY E. ZAIKOV
AND
LARISA P. KRYLOVA
EDITORS

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# PREFACE

This book presents current research in the fields of biochemistry and biotechnology. Topics discussed include information about the production of thermostable DNA polymerase suitable for whole-blood polymerase chain reaction; isolation, purification and some properties of L-Lysine α-Oxidase from Trichoderma sp. 6; production of nitric oxide by endothelial cells infected with herpes simplex virus type 1; optimization of the process of cultivation of recombinant Escherichia coli TBI protein producing strain; development of HRP-functionalized carbon-coated iron nanoparticales using arenediazonium tosilates; experimental approach to the induction of nonculturable state of Lactococcus lactis and callus culture technology of spring soft wheat stress tolerant varieties.

Chapter 1 - Using methods of genetic engineering the chimeric protein with additional N-terminal sequence-non-specific DNA binding domain of *Sulfolobus solfataricus* (SSO7D) was obtained on the basis of Klen*Taq* DNA polymerase (N-terminal 279 amino acids deletion variant of full length *Taq* DNA polymerase). Fermentation, isolation and purification of the target enzyme was performed in selected conditions. It was found that SSO7D-Klen*Taq* DNA polymerase retained stability at elevated blood concentrations (up to 20%, v/v) and it can be used as a component of analytical kits in clinical investigations.

Chapter 2 - The variety of oncological diseases triggers the elaboration of new anti tumor drugs. Considerable achievements in human cancer therapy were based on the different sensitivity of normal and tumor tissues to the lack of the essential growth factors. This fact caused the search for the new enzymes effecting some metabolic reactions essential for the growing cancer cells. The first enzyme, which was used in oncology, was L-asparaginase. The preparations of this enzyme from various sources differed in their properties, so that their immunological reactivity did not intersept. L-asparaginases were effective for the treatment of lymphoid tumors, but did not show activity against other types of tumors. Several other enzymes were investigated as the potential anti cancer agents, but presently they do not play a significant role in tumor therapy.

Chapter 3 - It was shown that recombinant IFN-α activates nitrite oxide production by primary culture of human vascular endothelium and the level of effects depends on compound concentrations. HSV-1 infection of endothelial cells at a MOI 0,1 resulted in fast activation NO synthesis, NO production was decreased at a low MOI. IFN-α pretreatment and following HSV-1 infection of endothelial cells resulted in reducing of NO production at 24 hours, it was noted inhibition of virus reproduction in cells at the same time; at 48 hours NO synthesis by cells was activated and at the same time it was noted both increasing of virus reproduction at

these experiments and activation effect of rec. IFN-α without virus infection at first experiments. It was considered that the mechanism of process noted points out compose multicomponent dependence of recombinant IFN-α effects, HSV-1 infection and NO production by culture of human vascular endothelium. Possibly the mechanism indicates pleiotropic intracellular transduction pathways influenced to iNOS expression in endothelial cells.

Chapter 4 - The effect of cultivation conditions on the yield of TBI protein in the process of biosynthesis of recombinant *E.coli* JM 103/pTBI strain was studed. Cultivation conditions were optimized by the composition of nutrient medium, the time of addition of the protein biosynthesis inducer and the rate of aeration in the process of cultivation in flasks and laboratory fermenters. It was shown that the yield of target protein increased in an optimized medium by two-fold as compared to the control. The qualitative and quantitative characteristics of TBI protein isolated from the optimized TB plus medium were better as compared to the control.

Chapter 5 - Isolation and purification method of intracellular aminoacylase *Escherichia coli* with use of ion-exchange chromatography on DEAE cellulose is developed. Ferment is obtained with 30% yield activity and 32-fold purified. Physico-chemical properties of investigating ferment is studied.

Chapter 6 - The present article describes the method of immobilization of biomolecules (Horseradish peroxidase) on carbon-coated iron nanoparticles using arenediazonium tosylates. As a result, a magnetically controlled complex of biomolecules and nanoparticles was created. The complex formation was confirmed by FT-IR spectroscopy. It has been shown that peroxidase attached to nanoparticles preserves its enzymatic activity.

Chapter 7 - Long-term exposure of microbial populations to physical, chemical and biological stresses and their combinations causes formation of viable but nonculturable (VBNC) cells. Detection of VBNC in the environment, food, and living organisms is a problem of high medical and biological significance. We have studied 3 bacteriocin producing strains of *Lactococcus lactis* ssp. *lactis* (194, 729 and MGU). Obtained data show that the examined strains are capable of entry into VBNC state within 2 weeks under carbohydrate starvation stress. However, synthesis of antimicrobial compounds (bacteriocins) remained unaltered.

Chapter 8 - Spring soft wheat varieties *in vitro* selection for drought, salinity and acidity tolerance technology for Siberian local breeding material was developed. Multiple regeneration phenomenon of wheat callus culture was investigated for the first time. Method of cereal crops test of acidity tolerance was improved.

Chapter 9 - This work studies interactions between a small globular protein and a relatively large protein with a conformationally variable chain using dynamic light scattering, differential scanning calorimetry, circular dichroism, fluorescence, and absorption measurements. It uses the dilute and semidilute system water/bovine serum albumin (BSA)/acid gelatin as a model. Gelatin molecules are able to form interpolymeric complexes with BSA in water at the temperatures above the temperature of the conformation transition and BSA/gelatin ratio ~6:1 (mole/mole). Interpolymer interaction leads to collapse gelatin macromolecules due to their lost of the total negative charge, partial stabilization of the secondary structure (increase the mean helix content), and stabilization of BSA molecules against thermo aggregation. At the same time, the thermo aggregation process of BSA molecules passes ahead of their thermodenaturation process.

Chapter 10 - We examine the issue of whether of low-volume fractions of nano- and microparticles can lead to phase separation in semidilute biopolymer mixture. To this end, we determine the phase diagrams and ESEM images of aqueous semidilute and weakly structured sodium caseinate-sodium alginate-dextran sulfate (SC-SA-DS) system in the presence of several the ultra clean charged micro particles with a diameter less, higher, and comparable with the size of the system network holes (2-3 um).We demonstrate that the last 3 um particles (both negatively and positively charged) help to enhance phase separation, and increase the viscoelastic properties of the emulsion, whereas the larger particles affect oppositely, and nanoparticles (210 nm and 910 nm in diameter) do not affect appreciably the phase separation and rheology. Experimental observations suggest that the dominant mechanism responsible for decrease thermodynamic compatibility in such system is perfect build 3 um particles into the holes (2-3 um in diameter) of the weak network of SC enriched phase and reinforcement of this network. Decrease in compatibility and increase in viscoelastisity are more pronounced by use highly charged hydrophobic carboxylate modified sulfonate latex (CLM-Sfn) and sulfate latex, and, in less degree, by use of aliphatic amino latex, and hydrophilic CLM latex.

Chapter 11 - The adsorption kinetics of 5-methylresorcinol (MR) at water-air interface after 2 and 24 hours incubation of MR solutions in 0.05M phosphate buffer with pH 7.4 at $25^{O}C$ was studied using a dynamic drop tensiometry via drop shape analysis techniques. The influence of methylresorcinol on adsorption of a model protein (hen egg lysozyme) was studied at fixed protein concentration $3.4\ 10^{-6}$ M and varying methylresorcinol concentration.

Chapter 12 - Application prospects of sporulating bacterial strain *Bacillus* subtilis for elaboration of new probiotic used in prevention and treatment of respiratory diseases in farm stock were demonstrated. Pilot-plant technology of Bacinil manufacturing and application was developed. Prophylactic and therapeutic efficiency of Bacinil in cases of enteritis and respiratory diseases of farm animals was established. Bacinil was registered by Vetbiopharm Council and entered into the list of veterinary drugs authorized for use in Belarus.

Chapter 13 - In the work presented the method for the prebiotics obtaining from common valerian extraction cake which remains after obtaining medicinal tincture.

Chapter 14 - Variations in physical-chemical properties of the block-copolymer of crab chitosan with methyl acrylate under the action of micromycetes (*Aspergillus terreus*) are investigated. The obtained data have been compared with the characteristics of chitosan and polymethyl acrylate as well as the mixture extracted from the solution of corresponding homopolymers. The standard enthalpy of combustion and chitosan formation have been determined.

Chapter 15 - The article covers the process of the biodestruction of block and grafted copolymers of chitosan with acrylic monomers (methylacrylate, acrylamide, acrylonitrile) under the action of various species of microscopic fungi and their single ferments: oxidoreductases (catalase, peroxidase) and hydrolases (chitosanase, protease, esterase). The most effective destructors for all the studied compositions are the following: Aspergillus niger, A. terreus, Chaetomium globosum, Penicillium cyclopium, P. funiculosum, Trichoderma viride. The example with the usage of chitosan copolymers with methylacrylate exhibits that the destruction process involves synthetic fragments of macrochains which can be concluded from a triple decrease of molecular mass and a drop in physical- mechanical properties of polymer mats. The molecular mass of the fragment of the synthetic polymer in

block and grafted (co)polymers is proved to be the limiting factor in the process of the design of totally biodegradable polymers.

Chapter 16 - Currently the brassinosteroid plant hormones has attracted attention of the scientists, who are engaged in the synthesis and extraction of natural compounds, studies of their biological properties, development of new specimens for agricultural needs, due to their extremely high biological activity. As per their structure brassinosteroids are close to steroid hormones of animals, they have specific influence on the cell membrane permeability, due to the system of adenylate cyclasa they have impact on the activity of definite enzymes (biocatalysts, ferments), regulating the metabolic process and functions of the cells, and directly on the functions of the cells genome. Concentrated in the growth segments of browses and pollen, brassinosteriods penetrate the organisms of invertebrates and vertebrates, causing different specific and general protective effects.

Chapter 17 - A current concern of modern medicine, pharmacology and cosmetology is the replacement of chemical antimicrobials by natural herbal ingredients. Some of these components are biologically active substances in plant extracts. These are concentrated extracts from plant materials. The effectiveness of these extracts is largely due to the presence of certain chemicals. These existing active substances have a diverse composition and belong to different classes of chemical compounds: flavonoids, terpenoids, glycosides, some saponins, resins, phenolics, volatile compounds and tannins, carbohydrates and minerals. The unique composition of complex and biologically active substances in fresh-water hydrophytes and geophytes indicates a wide range of application. These substances attract the particular interest of specialists in the field of cosmetology. We are currently studying a selection of individual components from the test extracts of plants. We will plan to use these substances in the preparation of cosmetic products.

Chapter 18 - Determination of the total content of endogenous antioxidants (AO) in seeds of the investigated cultures and sprouts was performed by an express method of initiated oxidation of hydrocarbon – cumene developed in the Institute of Chemical Physics RAS. The concentration of vitamin C in dry seeds and sprouts was obtained by a liquid-chromatographic method. These were found to lie in the range $1.1 \cdot 10^{-6} – 1.1 \cdot 10^{-5}$ M/kg in seeds. In sprouts of these cultures the range was $1.8 \cdot 10^{-5} – 1.1 \cdot 10^{-4}$ M/kg. The maximum content of endogenous AO in the sprout material of investigated cultures was $1.4 \cdot 10^{-2} – 2.1 \cdot 10^{-1}$ M/kg.

Chapter 19 - Filamentous fungi mainly represented by species of *Aspergillus* and *Penicillium* genera were isolated from waste biomass of higher water plants (HWP). It was established that density of *Aspergillus* species isolated from biomass of HWP cultivated on sewages was 2-3 times higher compared to biomass of HWP cultivated on fresh water. Micromycetes possessing cellulase activity and natural resistance to certain pollutants were determined.

Chapter 20 - Model tests on iron removal from primary kaolin of Angren deposit with application of Acidithiobacillus ferrooxidans K-1 and silicate microorganisms by two-stage scheme were conducted. Decrease of iron content in form FeO till 0.2% and $Fe_2O_3$ till 0.27% was established. Application of microorganisms in purification processes of kaolin of AKS grade allowed to decrease content of $Fe_2O_3$ till 0.23% and to improve its technological properties.

Chapter 21 - Treatment of cotton seeds with Verbactin stimulated growth and development of useful soil borne bacteria enriching root system with active metabolites that

positively impacted both seeds' germination, growth and development of cotton plants. It was determined that Verbactin activated community of soil borne microorganisms, mineralizing organic and mineral compounds, transformating of soil humus, including ammonificating and oligonitrophilic bacteria, Azotobacter, denitrifying bacteria, actynomycetes and filamentous fungi.

Chapter 22 - In connection with the great construction of the Olympic objects in Sochi, while training students in the specialization of "Jurisprudence" and studying the discipline of "The Ecological right " there has arisen a sharp necessity to offer additional courses through practical lessons. Within the framework of these course students of the faculty of law attend mandatory training and study ecological problems in concrete conditions of the Olympic construction in Sochi. It will allow further training of highly qualified specialists who will be capable of successfully influencing a complex ecological situation in Sochi while promoting the ecological legislation, and taking into account, the recent infringements in this branch.

Chapter 23 - We conducted studies to develop methods of introducing the culture *in vitro* micropropagation and grown them in the Black Sea city of Sochi. The valuable plant Mentha longifolia (L.) Huds. Apical stem tips (0,2-0,3 mm) were planted on MS basal medium contained of MS salts, 100 mg $L^{-1}$ myo-inositol, 0.2 mg $L^{-1}$ thiamine HCl, 0.1 mg $L^{-1}$ pyridoxine HCl, 0.2 mg $L^{-1}$ ascorbic acid, 0,5 mg $L^{-1}$ nicotinic acid, 20 g $L^{-1}$ sucrose, and 0.6% cell culture reagent agar. Four to five weeks after initial culture, shoots were selected for our regeneration experiment.

Chapter 24 - On the occasion of the XXII Olympic Games 2014 Sochi is undergoing extensive reconstruction. Parks, boulevards, and federal and local roads felled for new construction, buildings and private houses knocked down. The less valuable flora as well as unique exotic, rare and endangered plants are died under the ax. A compensation program provides to minimize the damage. However, we can't evaluate the integrity of biodiversity, protected areas and most important thing is we don't know how long it takes the environment to heal such profound changes. So we ought to involve all the latest scientific and technical developments in full use for the conservation and breeding the most valuable of unique plants and attracting to the area of landscape design the local flora's diversity, because the North Caucasus has no equal richness flora diversity throughout the Russian Federation.

Chapter 25 - At the Sochi Institute of People's Friendship University, the opportunities of gaining a deeper understanding of biological disciplines' studies are furthered. The faculty of Biology was formed at the Department of Physiology. Necessary additions to the educational process will be incorporated according to the new directions among the other professional disciplines which will be the studying of methods of biotechnology and the broadening of opportunities of making new methods to protect endangered plants on a new scale.

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*Chapter 1*

# PRODUCTION OF THERMOSTABLE DNA POLYMERASE SUITABLE FOR WHOLE-BLOOD POLYMERASE CHAIN REACTION

***A. S. Korovashkina, S. V. Kvach, L. A. Eroshevskaya and A. I. Zinchenko****
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## ABSTRACT

Using methods of genetic engineering the chimeric protein with additional N-terminal sequence-non-specific DNA binding domain of *Sulfolobus solfataricus* (SSO7D) was obtained on the basis of Klen*Taq* DNA polymerase (N-terminal 279 amino acids deletion variant of full length *Taq* DNA polymerase). Fermentation, isolation and purification of the target enzyme was performed in selected conditions. It was found that SSO7D-Klen*Taq* DNA polymerase retained stability at elevated blood concentrations (up to 20%, v/v) and it can be used as a component of analytical kits in clinical investigations.

**Keywords:** Polymerase chain reaction, plasmid, induction of protein expression, pET system, SSO7D-Klen*Taq* DNA polymerase, sequence-non-specific DNA binding domain, Escherichia coli

## 1. INTRODUCTION

Polymerase chain reaction (PCR) is a simple, efficient, and economical method for amplifying a small amount of DNA template but purified DNA is required [1, 2]. Blood as a typical biological sample is widely used in PCR-based studies, in diagnosis of microbial infections and genetic diseases, as well as in forensic analyses and blood banking [3, 4].

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Widely used DNA polymerases, such as *Taq* DNA polymerase and its analogs can be completely inactivated with 0.2% of whole human blood [5, 6]. Since DNA extraction is costly and time consuming, creation of DNA polymerase resistant to high concentrations of whole-blood would be very beneficial.

To overcome some of above-mentioned shortcomings of DNA polymerases a special approach was proposed in literature aimed at production of chimeric proteins incorporating in addition to polymerase domain a non-specific DNA-binding domain enhancing matrix affinity of polymerase and, as a result, increasing processivity, synthesis rate, precision and resistance to high ionic strength of solution [5, 7, 8].

In this study we have engineered *Escherichia coli* strain producing the chimeric protein which contains Klen*Taq* DNA polymerase (N-terminal 279 amino acids deletion variant of full length *Taq* DNA polymerase) and additional N-terminal sequence-non-specific DNA binding domain of *Sulfolobus solfataricus* (SSO7D).

## 2. MATERIALS AND METHODS

The fragment of Klen*Taq* DNA polymerase gene (KT) (truncated form of full-length 832 amino acid *Taq* polymerase lacking the N-terminal 279 amino acid portion) was amplified by PCR from the chromosomal DNA of *Thermus aquaticus* (ATCC 25104) using the following synthetic oligonucleotide primers: F (5'-*GAATTC*CTCCTCCACGAGTTCGGCCTTC-3') и R (5'-TAT*GTCGAC*TTAGTGATGGTGATGGTGATGCTCCTTGGCGGAGAGCCAGT-3'). *Eco*RI (F) and *Sal*I (R) restriction sites were inserted at 5′ ends of the primers (the bases are in italic). The R oligonucleotide contains an additional sequence (shown in bold) encoding a 6-His affinity tag at the C-terminus of resulting protein to facilitate its purification.

Amplification was performed using *Taq* or *Pfu* DNA polymerase and a PTC-200 GeneAmp PCR system from Bio-Rad (USA). The resulting products were isolated with Wizard SV gel and PCR clean-up system (Promega, USA), digested with *Eco*RI and *Sal*I restriction endonucleases, and ligated into the pET22b+ (Novagen, USA) expression vector, which had been restricted with the same enzymes. The resulting recombinant plasmid was designated pET22KT.

The nucleotide sequence encoding protein SSO7D (gene *sso7d*) was obtained from NCBI GenBank [9] (gene ID: 1453539, downloaded in October 2009). Codons for protein expression in *E. coli* cells were designed using DNA 2.0 Gene Designer (USA). The restriction site for *Nde*I was linked to the 5' end of the gene *sso7d*. The 3' end was elongated with nucleotide sequence, encoding linker oligopeptide and restriction sites for *Eco*RI. Design of oligonucleotides to assemble the gene, coding for protein SSO7D, was carried out using program TmPrime, version 3.0 (http://prime.ibn.a-star.edu.sg). Assembling of the SSO7D domain was completed by PCR [10].

The resulting PCR product (gene *sso7d*) was digested with *Nde*I and *Eco*RI restriction endonucleases and ligated into the pET22KT vector, which had been restricted with the same enzymes.

The resultant recombinant plasmid (designated pET22KT-SSO7D) was introduced into *E. coli* strain BL21(DE3). The transformed cells were grown at 37°C with orbital shaking at 200 rpm in 250 ml Erlenmeyer flasks containing 50 ml of Luria-Bertani (LB) culture

medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl in deionized water adjusted to pH 7 with KOH. Cultivation was continued until the optical density at 600 nm reached 0.6. Then isopropyl-β-thiogalactopyranoside (IPTG) was added up to concentration of 1 mM and fermentation was carried out for another 5 h. Cells were harvested by centrifugation for 10 min at 12,000 × g, washed once with 30 mM potassium phosphate buffer (pH 7.0) and slurried in the buffer, consisting of 50 mM $NaH_2PO_4$, 300 mM NaCl and 20 mM imidazole (pH 8.0).

The cells were sonicated twice at 4°C for 2.5 min and heated at 70°C for 30 min. After centrifugation of the sample for 30 min at 20,000 × g, the SSO7D -Klen*Taq* DNA polymerase was isolated from supernatant, using metal affinity chromatography with Ni-NTA agarose (Qiagen, USA). The resulting enzyme solution was dialyzed overnight against several changes of 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol and 0.5% Tween 20.

The dialyzed protein sample was dissolved three-fold in 10 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol, 0.5% Tween 20 and 50% glycerol.

The activity of SSO7D-Klen*Taq* DNA polymerase was determined using PCR. Amplification by PCR was performed in a reaction mixture (30 μl) containing 5 ng of plasmid DNA as a template, synthetic primers (5 pmol each), F 5'-GTCTACCAGGCATTCGCTTCAT-3' and R 5'-CTGTGA ATGCTGCGACTACGAT-3'), four deoxyribonucleoside triphosphates (0.2 mM each), $MgCl_2$ (3 мМ), KCl (90 mM), 50 mM Tris-HCl buffer (pH 8.8), 0.05% Tween 20 and 1 μl of SSO7D-Klen*Taq* DNA polymerase solution. The reaction was conducted for 30 cycles (15 s at 95°C, 15 s at 65°C and 30 s at 72°C). The 3 μl sample of the PCR mixture was then added to 200 μl of DNA-intercalating dye SYBR Green I (10,0000X stock solution, Sigma, USA) diluted 1:1600 in TE buffer, and fluorescence was measured using Qubit fluorometer (Invitrogen, USA). The amount of SSO7D-Klen*Taq* DNA polymerase corresponding to 1 unit of *Taq* DNA polymerase (Sileks, Russia) was assumed to be equivalent to one unit.

## 3. RESULTS AND DISCUSSION

It is known from literature reports that SSO7D domain allows to raise considerably affinity of DNA polymerase to the matrix and, as consequence, to increase processivity, synthesis rate, resistance to high ionic strength and various inhibitors [8]. Based on these data we presumed that chimeric DNA polymerase derived in the course of this research will be also more resistant to whole-blood inhibition. To assess resistance of SSO7D-Klen*Taq* DNA polymerase to whole blood we performed analysis of single point mutation in gene encoding blood coagulation factor V (FV, FV Q506, or FV Leiden). Presence in this gene of G→A substitution at nucleotide 1691 position is reported to bring up risk of venous thromboembolism in heterozygotes 8-fold, in homozygotes – 80 or even 100 fold [11]. Although only 5% of adult population are carriers of mutant allele, this mutation is detected in 40% of patients suffering from venous thrombosis. It follows that early screening for this mutation is essential for accurate diagnosing and formulation of proper treatment strategy. To evaluate effect of EDTA-stabilized blood on PCR engaging our original SSO7D-Klen*Taq*

DNA polymerase, blood of healthy adults was supplied into reaction mixture at various concentrations (1–20%, v/v). For amplification of FV gene segment containing nucleotide 1691, synthetic primers (F 5'-TGCCCAGTGCTTAACAAGACCA-3' и R 5'-TGTTATCACA CTGGTGCTAA-3') were applied [11]. The obtained data were analyzed using agarose gel electrophoresis (Figure 1).

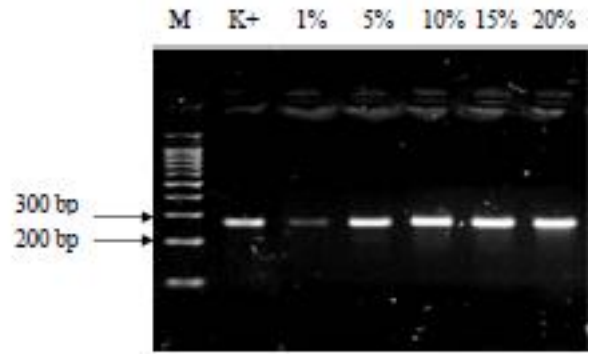


Figure 1. Electrophoregram of amplified FV gene segment containing nucleotide 1691. M, DNA molecular size markers; K+, PCR product of FV gene segment using 10 ng of purified DNA; 1–20%, whole blood concentration in the sample.

It is evident from electrophoregram data that the PCR product has the size approximately equal to 267 bp corresponding to theoretical estimates. It may be concluded therefore that SSO7D-Klen*Taq* DNA polymerase displays high enzyme activity even in the presence of elevated concentrations of whole blood (up to 20%, v/v). Presence of single point mutation was checked upon treatment of amplified samples with restriction endonuclease *Mnl*I. It was shown earlier that restriction of wild-type allele generates products 67, 37 and 163 bp long whereas the enzymatic cleavage of mutant allele yields fragments of 67 and 200 bp size. Analysis of derived fragments was performed by agarose gel electrophoresis (Figure 2).

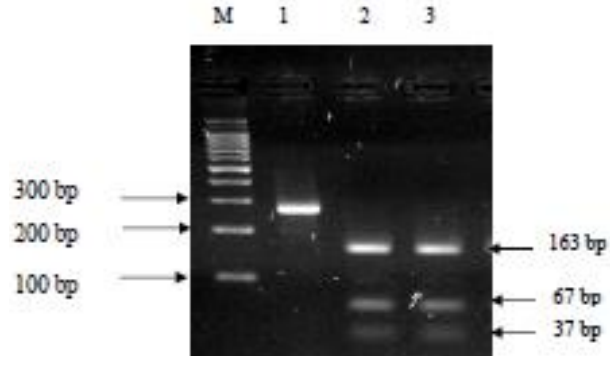


Figure 2. Electrophoregram of restriction fragment length polymorphism analysis of amplified FV gene segment containing nucleotide 1691. Line M is DNA molecular size markers; line 1 is the untreated segment; lines 2 and 3 are *Mnl*I restriction fragments of FV gene segments from two individuals.

The electrophoregram shows that samples from both individuals contain DNA fragments sized 37, 67 and 163 bp matching wild-type allele of the gene encoding FV. As a result such patients are homozygous for wild-type allele of FV. Summing up, it was demonstrated that

engineered chimeric enzyme SSO7D-Klen*Taq* DNA polymerase may withstand high whole blood concentrations (up to 20%, v/v) and may be applied as a constituent of analytical test-kits in clinical-laboratory investigations.

## ACKNOWLEDGMENTS

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*Chapter 2*

# ISOLATION, PURIFICATION AND SOME PROPERTIES OF L-LYSINE α-OXIDASE FROM TRICHODERMA SP. 6

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## ABSTRACT

The variety of oncological diseases triggers the elaboration of new anti tumor drugs. Considerable achievements in human cancer therapy were based on the different sensitivity of normal and tumor tissues to the lack of the essential growth factors. This fact caused the search for the new enzymes effecting some metabolic reactions essential for the growing cancer cells. The first enzyme, which was used in oncology, was L-asparaginase [1]. The preparations of this enzyme from various sources differed in their properties, so that their immunological reactivity did not intersept. L-asparaginases were effective for the treatment of lymphoid tumors, but did not show activity against other types of tumors. Several other enzymes were investigated as the potential anti cancer agents, but presently they do not play a significant role in tumor therapy [2].

It is well known that L-lysine is essential for human organism because there are no reactions for its biosynthesis, so food is the only source of this amino acid. L-Lysine is very important for biochemical processes. It stabilizes deoxy hemoglobin; the DNA-binding proteins of chromatin are rich with lysine as well as the proteins of connective tissues. Tumor cells are more sensitive to the lack of essential growth factors in comparison with the normal cells. The depletion of L-lysine causes inhibition of tumor cells growth. One of the enzymes destroying L-lysine is L-lysine alpha-oxidase (1.4.3.14) (LO), which was isolated for the first time in Japan [3] from the *Trichoderma viride* Y-244-2 grown on soaked wheat bran. Later LO was isolated in Russia from the strain *Trichoderma harzianum* Rifai [4]. For the purposes of practical oncology it is very important to have the enzymes, which catalyse the same reaction, but differ in their

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immunological reactivity. This aim could be achieved by using different sources of enzyme.

The aim of the present work was to find new strains-producers of LO, capable of intensive enzyme biosynthesis in the bioreactors, and to investigate LO from the new strain with the purpose of its practical application in oncology.

## MATERIAL AND METHODS

Microorganisms. Genus *Trichoderma* fungi were tested for their ability to synthesize LO. 12 strains under study were from the All-Russian Culture Collection of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences: *T. harzianum* Rifai VKM F-1959, *T. longibrachiatum* Rifai VKM F-2025, *T. virens* (J.H. Mill., Giddens and A.A. Foster) Arx VKM F-1117, *T. aureoviride* Rifai VKM F-2026, F-2027, *T. viride* Pers. VKM F-2721, F-1130, F-1132, F-1133, F-1134, F–1135, F–2430. 8 strains were isolated from soil (fields near Pushchino, Moscow region).

The isolation of the strains from soil samples. Enriched cultures were obtained on dry sterile wheat bran (a few passages). Soil samples were suspended in sterile distilled water and added to sterile dry wheat bran (10g). After 5 days of cultivation at 28 $^{o}$C 100 ml of sterile water were added into each flask. After shaking for 1 h aliquots were inoculated to an agar medium, containing the crushed wheat bran (10%) and $NaNO_3$ (5%). The grown up colonies were treated by the solution containing L-lysine (2.0 мМ), o-dianizidine (0.1 мМ) and peroxidase (5 μg/ml). Fungi from the colored (brownish) colonies were tested *again* for their ability to synthesize LO under solid-phase fermentation.

The solid-phase cultivation of the fungi. Fungi from colored colonies as well as museum strains were grown at 28$^0$C under periodical shaking in the 750-ml Erlenmeyer flasks with 10 g of sterilized bran and 10 ml of 4% solution of different nitrogen salts (or distilled water) (media N 1). The cultivation was carried out for 15 days. After fermentation 100 ml of Tris-HCl buffer (25 мМ, pH 8.0) were added to each flask and the mycelium with wheat bran were shaked for 1 hour (220 rpm). The insoluble residue was removed by centrifugation at 10000 g for 10 min and supernatant was used for enzyme isolation and purification.

The bulk cultivation of the fungi. Fungi were grown at 28$^0$C on a shaker (200 rpm) in 750-ml Erlenmeyer flasks with 100 ml of a Capek's medium, containing (g/l): $NaN0_3$ – 4.0; $KH_2PO_4$-1.0; $MgSO_4$-0.5; KCl–0.5 $FeSO_4$-0.01, and supplemented with Burkholder trace elements solution and the sole source of carbon and energy such as glucose (5.0%) (medium 2a), or starch (5.0%) (medium 2b), or sucrose (5.0%) (medium 2c), or glucose (1.0%) and corn extract (1.0 %) simultaneously (medium 2d). The pH of the media was 7.2.

The fungi were grown also on the liquid media, containing 10 g of wheat bran per 100 ml of distilled water and supplied with different nitrogen sources (media 3). After fermentation the cultural liquid was centrifuged (10000 g for10 min). The insoluble residue was removed and supernatant was used for enzyme isolation and purification.

LO biosynthesis monitoring. The concentration of LO in the reaction media was tested every day. To determine LO activity the aliquots of growing media were taken, filtrated and centrifuged at 10000g for 10 min.

LO activity was assayed at 25 oC by measuring the velocity of hydrogen peroxide formation in 20 mM Tris-HCl buffer (pH 8.0) in the presence of o-dianisidine (0.2 mM), peroxidase (5 μg/ml) and L-lysine (2.0 mM) on "Shimadzu" spectrophotometer (E436 = 11,0 mM-1·sm-1) [5].

Substrate specifity of LO was determined under the same conditions. 0.5 мМ L-lysine, L-leucine, L-phenylalanine, L-citrulline, L-alanine, L-asparagine, L-histidine, L-ornithine, L-glutamine, L-threonine, L-tyrosine, L-isoleucine, L-valine, glycine and D-lysine were used as the substrates, LO was added at the concentration equal to 2.0 μg/ml .

pH-dependence of the velocity of L-lysine enzymatic oxidation was measured under the described above conditions using 50 mM buffer solutions: formiate-acetate (pH 4.0-6.0), Tris-acetate (pH 6.0-7.5); Tris-HCl (pH 7.0-9.0).

The LO thermostability was studied in 2.5 mM Tris-HCl buffer by incubation of 2.0 μg/ml enzyme solution during 10 min at 20, 28, 37, 50, 60, 70 80$^{o}$C before enzymatic activity determination.

LO stability at 37$^{o}$C was studied by incubation of 5 mg/ml enzyme solution during 5 days. The aliquots were taken periodically for enzymatic activity determination.

The effectiveness of biosynthesis was estimated as the amount of International Units of LO activity per 1 ml of fermentation mixture (U/ml) or per 1 g of dry wheat bran used for fermentation (U/g). (It should be noted that fungal mycelia as usual grow penetrating through the wheat bran, so it was not possible to withdraw both the biomass and all the proteins including LO from the swallen solid substrate and to determine the ratio of U/per g of protein).

LO isolation and purification. Stage 1. After the filtration and centrifugation of cultural liquid the ballast proteins were removed during one day at 4 $^{o}$C by ammonium sulfate addition up to 25% of saturation and subsequent centrifugation (6000 g, 40 min). Ammonium sulfate was again added to the supernatant up to 55% of saturation and the solution was incubated at 4$^{o}$C for 12 hours.

Stage 2. The insoluble proteins, including LO, were separated by centrifugation (6000 g, 30 min) and dissolved in tris-HCl buffer (50 мМ, pH 8.0), containing ammonium sulfate (20% of saturation). The obtained solution was centrifuged to withdraw the insoluble proteins and applied to the column (2 x 30 cm) with Octyl-Sepharose CL-4B, equilibrated with tris-HCl buffer (25 мМ, pH 8.0), containing ammonium sulfate (20% of saturation). The column was washed with the same buffer. The enzyme was eluted by stepwise lowering the degree of ammonium sulfate saturation: 20, 15 and 10%.

Stage 3. The fractions with LO activity were collected, united, dialyzed during 12 hours against tris-HCl buffer (50 мМ, pH 8.0) and applied to the the column (2 x 40 cm) with DEAE-Toyopearl, equilibrated and washed by tris-HCl buffer (25 mM, pH 8.0). The enzyme was eluted by the fractional enhancement of NaCl concentrations: 0.15; 0.2; и 0.3 M.

Molecular weight and purity of the enzyme were determined by native electrophoresis [6] using PROTEAN II xi system BIO-RAD (USA). 50 μl analyzed samples containing 50 μg of protein were added into 50 μl of SDS sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 1.0% ß-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue). 20-25 μl of protein samples were applied on the gel for analysis.

After electrophoresis the gel was stained by Coomassie Brilliant Blue R250.

To define better the associated minor proteins a more sensitive method - silver staining was used.

Molecular weight determination by gel filtration was fulfilled on the colomn with Toyopearl HW-55 (1,5 x 95 cm). The elution was carried out by Tris-HCl buffer (25 mM, pH 7.8). Alcohol dehydrogenase (150 κDa), bovine serum albumen (67 κDa), *a*-chymotrypsin (25 κDa) and cytochrome *c* (13 κDa) were used for column calibration. $V_0$ of the column was measured with the help of Blue Dextran 2000.

Cofactor analysis. Aqueous solution containing LO (100 μg/ml) and trichloracetic acid (10%)

kept for 5 min at 100 $^{o}$C. The denaturized proteins were withdrawn by centrifugation (10000 g, for 10 min). The supernatant was neutralized by KOH and used for the measurement of optical density at 460 nm (ε=11300 x $mol^{-1}$ x $cm^{-1}$) (Shimadzu). Qualitative analysis of the cofactor was carried out with HPLC, colomn Separon $C_{18,}$ 4x150, 5 μm (Czech Republic).

## RESULTS AND DISCUSSION

Genus *Trichoderma* fungi were tested for their ability to produce LO using solid-phase cultivation method. All strains were actively growing on wheat bran (medium 1), but only 4 strains appeared to produce LO, the highest LO level being shown by *Trichoderma* sp. 6 (Table 1). The addition of different nitrogen sources enhanced the LO biosynthesis 5-10 times.

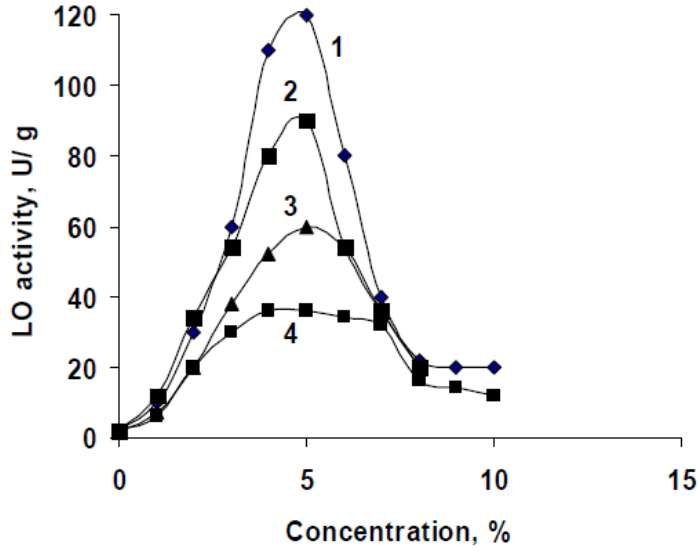
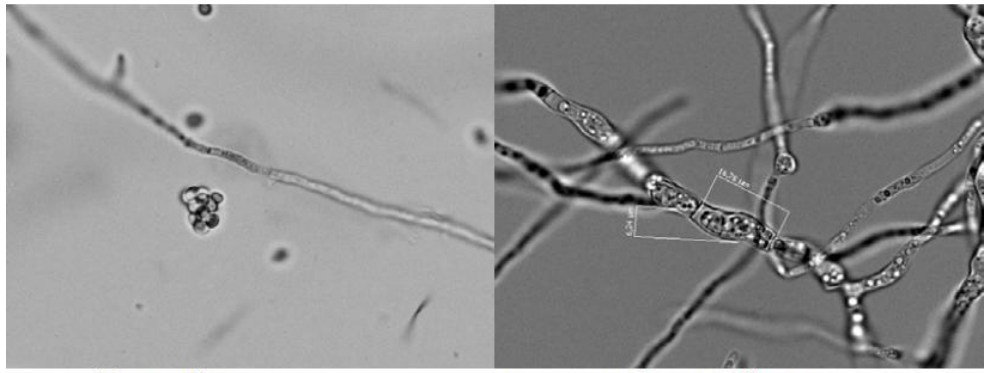



Figure 1. The influence of nitrogen sources on LO synthesis by *Trichoderma* sp. 6. 1 - $NH_4NO_3$; 2 - $NH_4Cl$; 3 - NaNO3; 4 - $(NH_4)_2SO_4$.

The bulk cultivation of four mentioned fungi strains using the media and conditions described in “MATERIAL AND METHODS showed that all strains were growing intensively in the used media (visual observation), including the Capek’s medium with

different carbon sources: glucose, starch or sucrose (media 2, 3). But LO biosynthesis was detected only in the media containing wheat bran (medium 3). The highest LO activity was also found when *Trichoderma* sp. 6 was used. The enzyme biosynthesis depended gradually from the presence of nitrogen sources, The best results were obtained in the presence of 6% $NH_4NO_3$: LO activity in the medium reached 8.5 U/ml (120 U/g – wheat bran (Figure 1). It was twice higher than LO level reached using solid-phase cultivation method. The dependence of LO biosynthesis from the conditions of cultivation was noticed for the first time by the Japanese scientists for the *Trichoderma viride* Y-244 strain: the addition of $NaNO_3$ during solid-phase fermentation increased LO yield 3 times, but in bulk conditions this strain was not able to produce LO [3]. Strain *T. harzianum* Rifai was able to synthesize LO during surface and bulk cultivation, but the enzyme levels were rather low [7].



A – aerial mycelium (solid-substrate cultivation)

B - vegetative mycelium (bulk cultivation).

Figure 2. Microphotographs (magnification x 63) of *Trichoderma* sp.6, producing L-lysine α-oxidase.

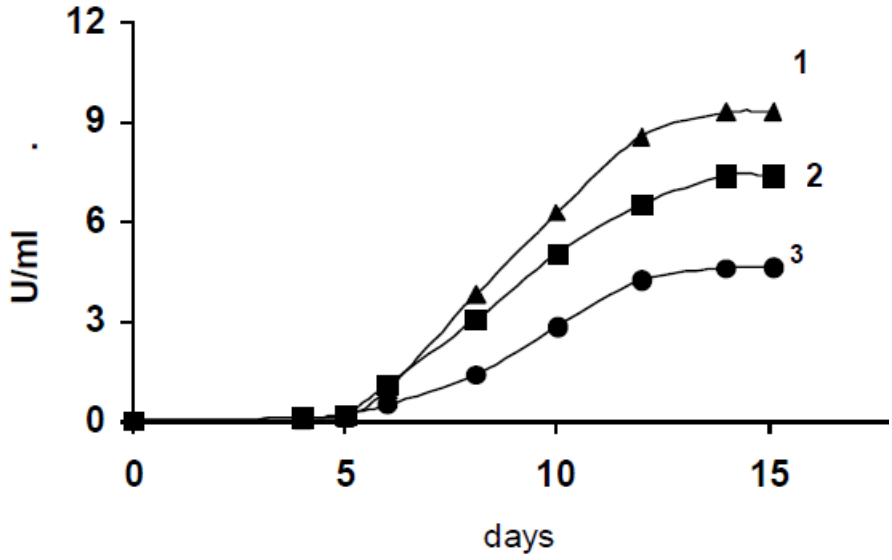



Figure 3. Influence of aeration on LO synthesis by *Trichoderma* sp. 6 in bioreactors. Temperature -28° C, pH – without adjustment. 1. - $pO_2$ 80%; 2.- $pO_2$ 55%; 3.- $pO_2$ 30%.

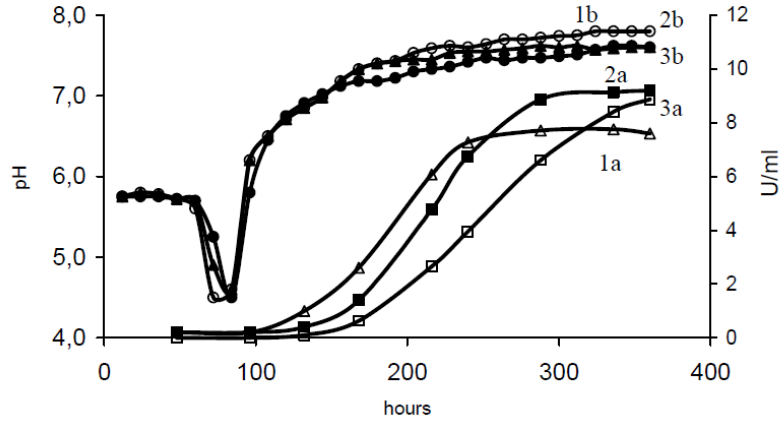



Figure 4. Influence of the temperature on LO synthesis by *Trichoderma* sp. 6 in bioreactors. $pO_2$ -70-80%, pH – without adjustment. a-LO activity; b- pH. -37 °C; 2. -29 °C; 3. -24 °C.

Microphotographs of *Trichoderma* sp. 6, grown on wheat bran by solid phase fermentation (A) and bulk cultivation (B) are presented in Figure 2. The morphological properties of this strain are similar with the strains *T. viride* Y244-2 and IFO 4847, capable to synthesize LO [8], but not so intensively as the original strain *Trichoderma* sp. 6, which was found in the present work.

**Table 1. Synthesis of extra cellular L-lysine α-oxidase (U/g of wheat bran)* by *Trichoderma* fungi during solid-phase cultivation. Influence of different nitrogen sources (4%)**

| Strain | Nitrogen sources | | | | | |
|---|---|---|---|---|---|---|
| | - | $NaNO_3$ | $KNO_3$ | $(NH_4)_2SO_4$ | $NH_4H_2PO_4$ | $NH_4NO_3$ |
| *T. aureoviride* Rifai VKM F-2026 | 2 | 15 | 10 | 10 | 10 | 20 |
| *T. viride* Pers. VKM F-2721 | 3 | 20 | 10 | 15 | 10 | 30 |
| *T. longibrachiatum* Rifai BKM F-2025 | 2 | 10 | 10 | 5 | 5 | 15 |
| *Trichoderma* sp. 6 | 5 | 30 | 20 | 20 | 15 | 50 |

*Maximal activity observed during cultivation on the 12-13 day.

As *Trichoderma* sp. 6 afforded to obtain the highest yield of LO during the solid-phase and bulk cultivation in the flasks, this strain was chosen for LO biosynthesis in large scales. The influence of aeration on LO biosynthesis was studied in 3-l bioreactors. The cultivation was carried out in the medium containing 6% $NH_4NO_3$, at 29°C without pH correction. The most active LO biosynthesis - 8.5 U/ml (120 U/g of wheat bran) occurred at $pO_2$ 70-80% of saturation (Figure 3, curve 1). At lower $pO_2$ the LO biosynthesis was not so high (curves 2, 3) probably because this levels of oxygen were insufficient for biomass growth. Figure 4. shows the influence of temperature on LO biosynthesis at controlled $pO_2$=70-80% of the saturation.

At the beginning of the cultivation the highest LO biosynthesis occurred at 37° C (curve 1a), but after 6-8 days the process slowed down and no further LO biosynthesis was observed. The highest LO yield was obtained at 29° C: on the 11-12 day up to 125 U/g wheat bran (curve 2a). At 24 °C (curve 3a) LO accumulation proceeded slowly LO and the total time of cultivation enhanced up to 16 days.

The influence of pH on LO biosynthesis was fulfilled at 29° C and $pO_2$ 70-80% of saturation. Without pH maintaining during the first 4 days the pH of the medium shifted to the acidic area pH 5.8 - 4.5 (figure 5), but later the pH value slow increase up to 7.8 (curve 1a) was observed which coincided with the appearance of LO activity. The maintenance of pH values not lower than 5.8 (curves 2, 3) did not improve the results. The highest LO biosynthesis - 170 U/g of wheat bran (10 U/ml) was obtained in the case when pH was hold not higher than 6.0 (curves 4, a and b).

So the fermentation in the worked out optimal conditions (pH not higher than 6.0, pO2=70-80%, temperature 29°C) made it possible to increase the LO biosynthesis and to obtain 170 U/g of wheat bran (10 U/ml). The analysis of literature data points that so high level of LO enzymatic activity was not observed beforehand.

A new improved technique of LO purification based on ammonium sulfate precipitation (25-55% of saturation), chromatography on octyl-sepharose and DEAE-Toyopearl was used. This approach made it possible to obtain homogeneous enzyme with high specific activity 99 U/mg (25°C) and good yield 66%.

High thermal stability of LO was shown: the enzyme retained its activity up to 50ºC. Optical absorption spectrum of LO are analogous to those of flavoproteins with maximums at 278, 390 and 465 nm (shoulder at 490 nm) [9]. The prosthetic group of the enzyme proved to be FAD and each subunit possesses one molecule of FAD. The pH optimum of L-lysine oxidation by LO from *Trichoderma* sp. 6 was determined to be 7.8-8.0. This data coinside with the information about the enzymes from the other sources [5,9]. LO is a stereo specific enzyme.

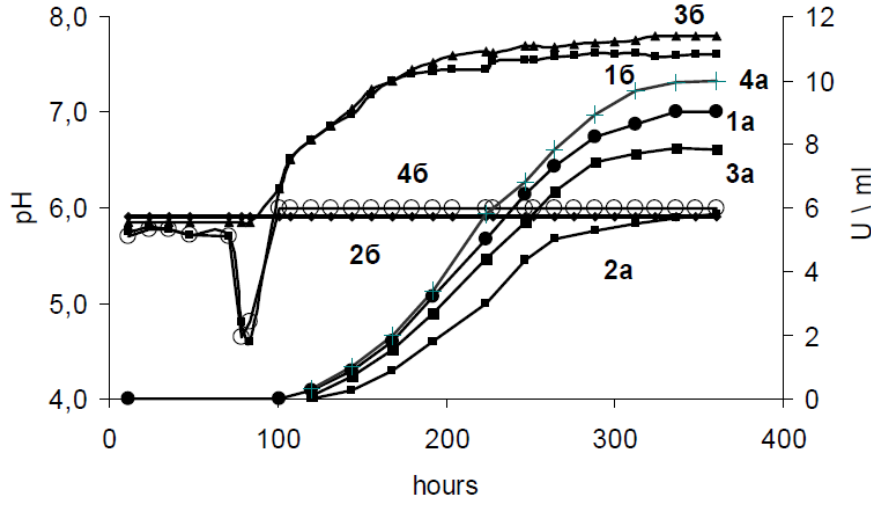



Figure 5. Influence of pH on LO synthesis by *Trichoderma* sp. 6 in bioreactors. $pO_2$ -70-80%, temperature -29 °C. a - LO activity; b - pH. 1. pH without corrtction; 2. pH 5.8; 3. pH ≥ 6.0; 4. pH ≤ 6.0.

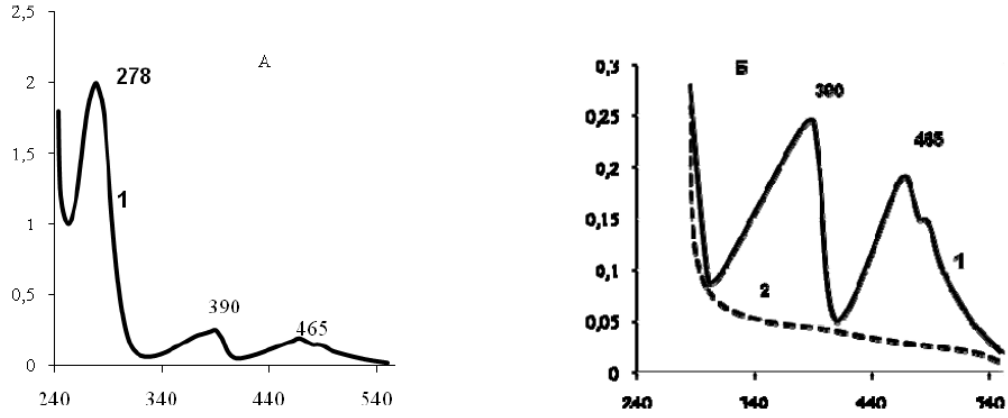



Figure 6. The optical absorbtion stectra of L-lysine alpha-oxidase (0.5 mg/ml LO in 25 mM Tris-HCl buffer pH 8.0). 1.- without L-lysine ; 2. - in the presence of 0.5 mM L-lysine.

It does not affect D-lysine. L-lysine is the main substrate of LO (100% activity). Among the tested amino acids (see MATERIAL AND METHODS) only structural analogs of L-lysine (L-ornithine and L-arginine) are oxidized, but much slower (less then 8%). Substrate specificity of LO from the new strain Trichoderma sp. 6 resembles the specificity amino acid oxidases from the other strains of genus *Trichoderma* (*T. viride* Y-244-2 [9] and *T. harzianum* Rifai [5] ). Molecular weight of enzyme determined by native electrophoreses and gel filtration was equal to 115-116 кDa. According to SDS electrophoresis data LO is a dimeric molecule with identical subunits of 57-58 kDa. High termal stability of LO was shown: the enzyme retained its activity up to 50 º C. The enzyme remained active at 37 ºC at least 5 days.

The spectra of optical absorption are presented in Figure 6. LO isolated demonstrated the spectra typical for flavin-containing proteins with maxima at 278, 390 и 465 nm. In the presence of L-lysine the maxima at 390 and 465 nm disappeared (figure 6, curve b). After exhaustion of lysine (5-6 min) spectra were recovered in the presence of oxygen. These data are indicating that flavin-containing coenzyme participates as the prosthetic group of LO. Further qualitative and quantitative analysis of the cofactor **(**see MATERIAL AND METHODS**)** showed that prosthetic group of the enzyme proved to be FAD and each LO subunit possessed one molecule of FAD. The biological properties of LO are mainly based on its catalytic activity: during the reaction L-lysine level is decreased and hydrogen peroxide formation occurs. L-lysine is an important molecule for cell growth and hydrogen peroxide presents one of the reactive oxygen species, which takes part in the oxidative stress development. LO antibacterial effect towards different strains of *Bacillus subtilis, Staphylococcus, Pseudomonas* and *Aspergillus* and anti fungal effect towards *Clostridium sporogenes* was shown. The strains of tumor cells are sensitive to the lack of L-lysine and to the action of oxidative stress caused by hydrogen peroxide.

The main difference between LO and L-asparaginase (the only enzyme used in tumor therapy) is that LO does not effect Fisher lymphadenosis L5178Y. Eight strains of tumor cells were shown to be sensitive to LO treatment. Currently further pre-clinical studies of LO anti tumor activity are carried out in Blokhin's Cancer Research Center of Russian Academy of Medical Sciences.

## CONCLUSION

L-Lysine α-oxidase (LO) presents one of the enzymes which are perspective for antitumor enzyme therapy. Its biological action is based on the depletion of essential amino acid L-lysine. During the cultivation on solid substrate (soaked wheat bran) of 12 different strains belonging to the genus *Trichoderma* the most intensive LO biosynthesis was observed with *Trichoderma*. sp. 6**.** The optimal fermentation conditions in laboratory bioreactors were elaborated ($pO_2$, pH, nitrogen sources and temperature), and it lead to the enhancement of LO biosynthesis up to 170 U/g of wheat bran. The analysis of literature data points that so high level of LO enzymatic activity was not observed beforehand. New effective technique of LO isolation and purification was developed and homogeneous enzyme preparation was obtained with specific activity 99 U/mg protein ($25^oC$). Molecular weight of enzyme determined by native electrophoreses and gel filtration was equal to 115-116 кDa. According to SDS electrophoresis data LO is a dimeric molecule with identical subunits of 57-58 kDa. Optical absorption spectrum of LO are analogous to those of flavoproteins with maximums at 278, 390 and 465 nm (shoulder at 490 nm). The prosthetic group of the enzyme proved to be FAD and each subunit possesses one molecule of FAD.

LO is a stereo specific enzyme oxidizing only L-lysine (pH optimum 7.8 – 8.0), insignificant activity towards L-ornithine and L-arginine was observed. High termal stability of LO was shown: the enzyme retained its activity up to 50 ºC. LO from a new strain producer exhibited cytotoxic, antibacterial and antifungal activity.

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*Chapter 3*

# PRODUCTION OF NITRIC OXIDE BY ENDOTHELIAL CELLS INFECTED WITH HERPES SIMPLEX VIRUS TYPE 1, UNDER THE INFLUENCE OF INTERFERON

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## ABSTRACT

It was shown that recombinant IFN-α activates nitrite oxide production by primary culture of human vascular endothelium and the level of effects depends on compound concentrations. HSV-1 infection of endothelial cells at a MOI 0,1 resulted in fast activation NO synthesis, NO production was decreased at a low MOI. IFN-α pretreatment and following HSV-1 infection of endothelial cells resulted in reducing of NO production at 24 hours, it was noted inhibition of virus reproduction in cells at the same time; at 48 hours NO synthesis by cells was activated and at the same time it was noted both increasing of virus reproduction at these experiments and activation effect of rec. IFN-α without virus infection at first experiments.

It was considered that the mechanism of process noted points out compose multicomponent dependence of recombinant IFN-α effects, HSV-1 infection and NO production by culture of human vascular endothelium. Possibly the mechanism indicates pleiotropic intracellular transduction pathways influenced to iNOS expression in endothelial cells.

**Kaywords:** Nitric oxide, endothelial cells, interferon alpha, herpes simplex virus type 1

## INTRODUCTION

It is by this time established that nitric oxide (NO), produced by vascular endothelium, possesses a great number of activities: is a major factor influencing a tone of vessels, takes part in inflammatory process and, besides, possesses antipathogenic action concerning a wide spectrum of infectious agents [3-5, 9, 18, 22]. NO is synthesized by endothelial cells of blood vessels (ECs) constitutively (eNOS) and at activation (iNOS) by physiological stimulators and vascular active constituents (6, 8, 10. Infringement of NO production by ECs lead to their dysfunction that is one of the basic pathogenesis steps of the most widespread diseases of cardiovascular system, including an atherosclerosis [11, 12, 14].

One of the first steps of atherogenesis is development of inflammatory process in a vascular wall which can express as a result of the influence of a great number of pathogens, including and herpesviruses [14]. Herpes simplex virus type 1 (HSV-1), the most widespread in human population a virus of herpes group, has been found out in biopsies of various bodies and atherosclerotic plaques [7, 12, 16]. Besides it is known that HSV-1 causes dysfunction in ECs in experiments in vitro [9, 18]. In clinics practice interferon alpha (IFN - α) is widely used at treatment of relapses of herpetic diseases [2]. The purpose of the study was in investigation of NO production by human EC culture under influence of human IFN - α, at an infection of EC culture with HSV-1 and joint influence on culture with IFN - α and HSV-1.

## MATERIALS AND METHODS

Endothelial cells (ECs) were allocated from an umbilical cord, received from healthy women after normal delivery according to standard protocols, described earlier [17]. Shortly: umbilical veins were filled by a solution of dispase (0,15 %, MP Biomedicals) and incubated within 30 minutes at temperature 37ºC. Then veins have been washed out by a physiological solution, cells have been collected from perfusion solution by centrifugation at 800 g within 5 minutes, resuspended in 199 medium (Gibco) with addition of 10 % fetal calf serum, endothelial growth factor, heparin (100 mcg/ml) and gentamycin (50 mcg/ml) and are transferred to plastic dishes. After formation of monolayer cells were resuspended in tripsin-EDTA (Gibco) and seeded with density 105 cells/$sm^2$ on 24 well plated . Only 4 days monolayer of ECs was used.

ECs were infected with herpes simplex virus type I (HSV-1), strain R39, received from WHO. Multipliciry of infection (MOI) was 0,1-0,0001 $TCID_{50}$/cell. Recombinant interferon IFN α2b was used ("Immunofarm", Russia). ECs were cultivated with IFN α in different concentration within 24 hours, cells were washed and infected with HSV-1, in dynamics samples of cultural medium were selected and samples before research were stored at-20°C. Levels of infectious virus in samples were tested by titration in monolayer of continuous cell line VERO seeded on 96 wells plates.

NO concentration was determined by the content of nitrite, which is the stable oxidation product of NO. The content of nitrite in the culture medium reflects the amount of synthesized NO. Quantity of nitrite was determined by the method of a standard Griess [10]. Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 1N HCl) and N-(1 - naphthyl) ethylenediamine dihydrochloride (NEDA) (0,15% in $H_2O$). 1 ml sample

was mixed with 1 ml of Griess reagent. ). Incubated for 20 min and measured the absorption of the chromophore at 540 nm on a spectrophotometer "Shimadzu" (Japan). $NaNO_2$ was used as a standard for constructing the calibration curve. In addition, used to determine the test system NO (R and D Systems) and Multiskan Antos 2020 at a wavelength of 540 nm. The experimental results are expressed in relative units. The EPR spectra of samples of culture medium, prepared as frozen columns with a diameter of 3 mm and a height of 30 mm, recorded on a spectrometer "ESP-300" firm "Bruker-Analitishe-Messtechnik" (Germany), at liquid nitrogen temperature. For the formation of NO followed by the appearance of the EPR spectra signals of nitrosyl complexes of hemoglobin (Hb), which was added as a trap NO.

## RESULTS AND DISCUSSION

Monolayer cultures of ECs were treated with IFN - α at concentration 1x106 - 1x105 IU/ml. Results of testing of nitrite are presented on figure 1. It has appeared that after treatment of ECs IFNα in concentration 1x105 IU/ml the amount of nitrite in cultural medium has increased to 10-15 % by 24 o'clock in comparison with its amount in control not treated IFN ECs. Treatment with IFNα in concentration 1x106 IU/ml resulted in to essential increase in the level of nitrite in cultural medium in comparison with interferon used in concentration 1x105 IU/ml. And, it is necessary to notice that increase of level of nitrite was observed already to 3 and 6 hours after using of IFN (approximately on 30 %), at the subsequent cultivation of ECs there was a further increase in the level of nitrite which to 24 and 48 hours exceeded control values on 50-60 %.

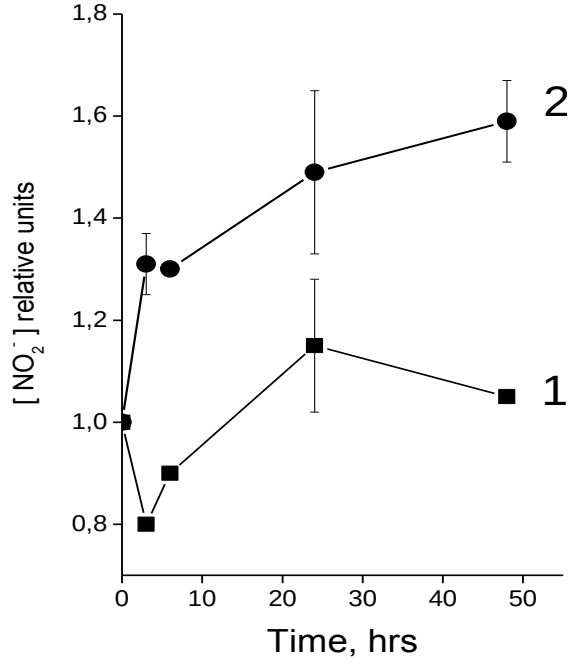


Figure 1. Dynamics of nitric oxide production in human vascular endothelial cells after treatment with interferon-α: 1 - concentration of IFN-α $1x10^5$ IU / ml, 2 - concentration of IFN-α - $1x10^6$ IU / ml.

Consequently, already after 3 hour contacts IFN - α (1x106 IU/ml) with ECs there was activation of oxide nitrogen production. Earlier it has been shown that activation of endothelial iNOS occurs earlier, than for other types of cells [15-16]. It has been established that levels мRNA for endothelial iNOS were maximum in 2 hours and further gradually decreased even at constant stimulation. At the same time it is known that macrophages мRNA for iNOS it is found out not earlier than in 4-6 hours and further synthesis NO only increases till 2-3 days in the presence of a substratum [1, 13]. This fast increase and the subsequent delay of synthesis iNOS in endothelial cells can reflect a functional difference between two types of cells – endothelial and macrophages making NO after stimulating agents effect.

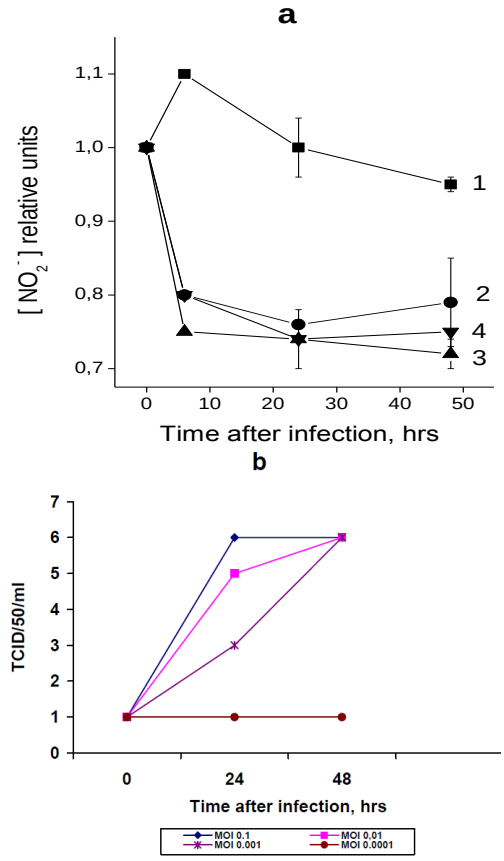


Figure 2. Production of oxide nitrogen (a) and a virus reproduction (b) in human vascular endothelial cells after infection with HSV-1 at MOI (TCID $_{50}$ /ml): 1 – 0,1; 2 - 0,01; 3 - 0,001 and 4 - 0,0001.

For the investigation of HSV-1 influence on ECs ability to synthesize nitric oxide monolayer cultures were infected with a virus with MOI 0,1-0,0001 TCID/$_{50}$. Selected samples of cultural medium of infected and control cells were tested. In samples the nitrites levels and an infectious virus were tested in dynamics. Results of experiments are presented on figure 2. From figure 2a it is visible that cells differently react to virus infection at different MOI. After infection at MOI 0,01, 0,001 and 0,0001 reduction of quantity of nitrite in cultural medium was observed: there was a decrease in synthesis NO by cells (figure 2, curves 2,3,4). At these MOI decrease in level of nitrite was 20-25 %. At an infection of cells

at the highest MOI (0,1) the small nitrite increase was observed (on 10-12 %) through 6 hours after infection, however in later terms decrease in formation NO was observed (Figure 2a, curve 1). From the results presented in figure 2b it is visible dose-dependence between MOI of virus and the level of an infectious virus in cultural medium of ECs: at more larger MOI it was shown fast and to higher titers of virus accumulation, at lower MOI virus accumulation was slower and values of virus titers were more low.

Increased NO production in cells infected with the virus at MOI 0.1 was somewhat unexpected. Although it is known that NO has antiviral effect [4, 5, 9, 22], however, a reproduction of the virus at this multiplicity was the highest. On the other hand, increased production of NO by ingestion of massive doses of virus can lead to vasodilation, slowing blood flow, accumulation of leucocytes, which may contribute to the development of long-term inflammation [15, 21].

Figure 3. shows the results of influence of pretreatment of ECs by IFN-α for 24 h and then infected with HSV-1 (MOI 0.1) on NO production and reproduction of the virus. It is seen that the joint effect of viruses and interferon in a concentration both $1x10^5$ IU / ml and $1x10^6$ IU / ml to 24 hours after infection leads to a decrease in nitrite content in culture medium by 30-40% compared with the content of nitrite in the control (intact) cultures. After 48 hours, with the use of IFN in a concentration $1x10^6$ IU / ml, a gradual recovery of nitrite content to values from 10 to 15% higher than in the control group was observed, whereas at the concentration $1x10^5$ IU / ml nitrite level remained low (Fig 3a).

The joint effect of the virus and interferon in a concentration both $1x10^5$ IU / ml and $1x10^6$ IU / ml through 24 hours resulted in significant reduction of viral titers in the culture medium ($10^{-1}$ −$10^{-1,5}$ $TCID_{50}$/cell) compared with their values in the culture medium of EC not treated with IFN. Virus titers in the culture medium of EC treated with IFN and infected with HSV-1 to 48 hours increased to a value of $1x10^{-4}$ and as in the control samples it was $10^{-6,5}$ $TCID_{50}$/cell (Figure 3b).

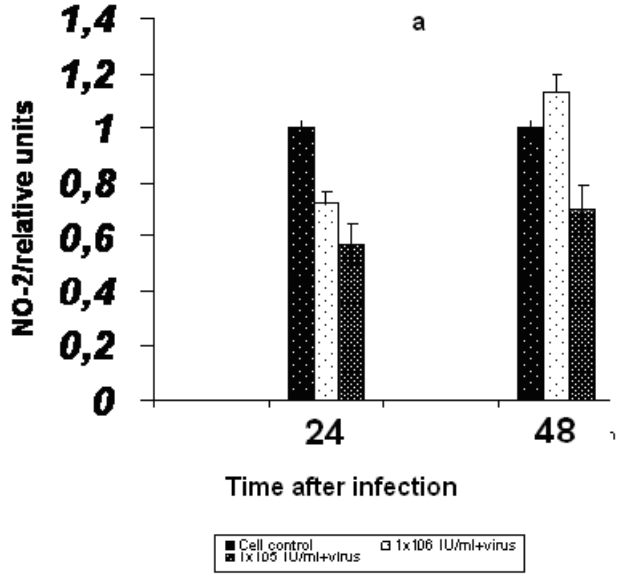


Figure 3. (Continued).

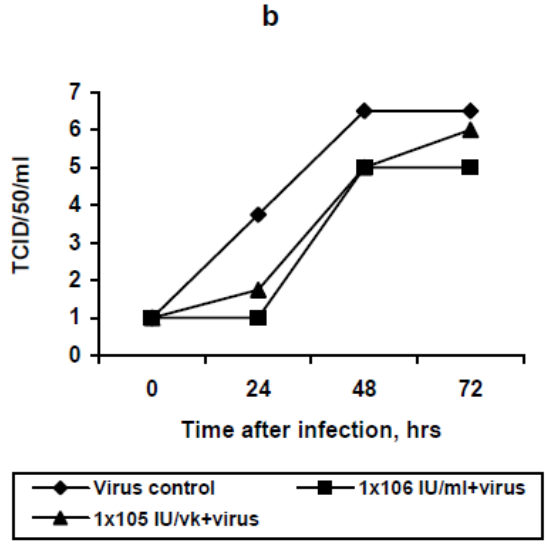


Figure 3. Change of nitric oxide production (a) and virus reproduction (b) in vascular endothelial cells after treatment with human interferon-α for 24 hours and subsequently infected with HSV-1 (MI, 0,1);

Reduction of NO production of IFN-treated and infected cultures can not be explained by the action of IFN, since in the absence of infection IFN at concentrations $1x10^5$ IU / ml and $1x10^6$ IU / ml caused an increase of NO production in ECs (Figure 1). Effect of HSV-1 at low MOI consisted in reducing nitric oxide synthesis by ECs (Figure 2). The connection between low levels of virus reproduction by endothelial cells under the influence of IFN to 24 hours (Figure 3a) and, consequently, low virus content, and decreased nitric oxide synthesis by these cells to 24 hours was observed (3a). The increase in virus reproduction in cells treated with IFN for 48 hours, and, consequently, a higher concentration of the virus, accompanied by increased synthesis of nitric oxide in ECs (Figure 3b). The proposed mechanism of this process indicates a complex relationship between the action of IFN-α, virus infection and NO production by endothelial culture. Probably it is due to the overlapping intracellular signaling mechanisms responsible for the effect of IFN-α and HSV on the expression of iNOS in endothelial cells. Nitric oxide synthesized by endothelial cells in the incubation medium also was detected by ESR. As the trap NO in this case, we used Hb, which has a high affinity for NO. Figure 4 shows EPR spectra recorded in samples of cell medium in which untreated EC were incubated for 24 h. Before preparing samples for EPR investigations into the incubation medium were added Hb ($5x10^{-6}$ M) as a trap NO and AK ($10^{-5}$ M) as a reducing nitrite ions.

In the observed EPR spectrum a well-known signal of nitrosyl complexes of hemoglobin with nitric oxide (NC Hb-NO) - a broad ESR signal with a characteristic triplet splitting at g-factor of 2.01 was recorded. These data indicate that NO secreted by endothelial cells, getting into the bloodstream, interacts with the molecules of Hb, displacing oxygen, which leads to the formation of nitrosyl complexes Hb-NO in blood. This method can be used to test the NO in the medium of cultured EC.

Thus, this study demonstrated that IFN-α activates nitric oxide production by cells of primary culture of human ECs of blood vessels and the value of the effect was depended on the concentration of the drug. Earlier, a similar concentration effect was observed in studying of the action of IFN-γ on ECs. It was shown that IFN-γ at concentrations up 1 mg / ml led to a

decrease in production of NO, at higher concentrations its action leads to a gradual increase in the synthesis of NO [18]. Infection of endothelial cells with HSV-1 at MOI 0.1 led to rapid activation of synthesis of NO, whereas at lower MOI nitric oxide production was decreased. Under the joint action of IFN-α (pretreatment) and HSV-1 was observed suppression of NO production by ECs to 24 hours, which coincided with inhibition of virus reproduction. Activation of nitric oxide synthesis to 48 hours coincided with both an increase in virus reproduction in this series of experiments, and with the activating effect of IFN-α in the absence of viral infection found in the first series of experiments.

Study was supported partly by Grant ISTC#3505.

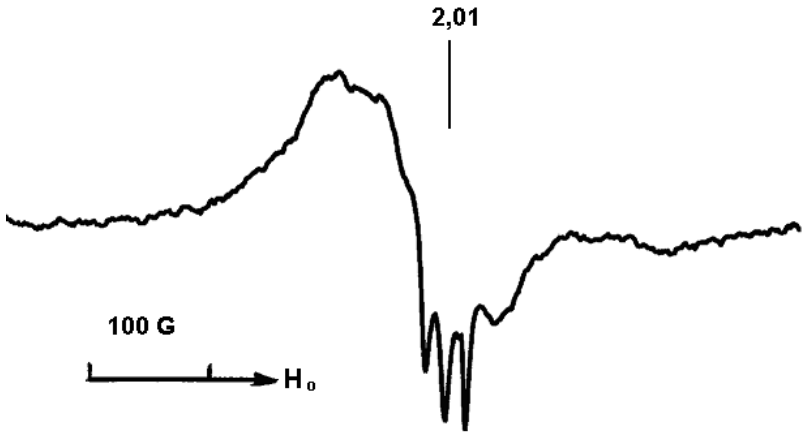


Figure 4. The EPR spectra of a sample of the culture medium of human vascular endothelial cells after cultivation with interferon-α for 24 h. After incubation, before preparing of the samples in medium were added ascorbate ($10^{-5}$ M) and hemoglobin ($5x10^{-6}$ M). Conditions of registration of EPR spectra: microwave power 20 mW, magnetic field modulation amplitude 4 G, the temperature measurement of 77 K.

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*Chapter 4*

# OPTIMIZATION OF THE PROCESS OF CULTIVATION OF RECOMBINANT *ESCHERICHIA COLI* TBI PROTEIN PRODUCING STRAIN

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## ABSTRACT

The effect of cultivation conditions on the yield of TBI protein in the process of biosynthesis of recombinant *E.coli* JM 103/pTBI strain was studed. Cultivation conditions were optimized by the composition of nutrient medium, the time of addition of the protein biosynthesis inducer and the rate of aeration in the process of cultivation in flasks and laboratory fermenters. It was shown that the yield of target protein increased in an optimized medium by two-fold as compared to the control. The qualitative and quantitative characteristics of TBI protein isolated from the optimized TB plus medium were better as compared to the control.

**Keywords:** Cultivation, recombinant strain, TBI protein, nalidixic acid, TB plus medium, biosynthesis, biomass, fermentation

## INTRODUCTION

The process of microbiological synthesis of TBI protein plays a key role in technology of production of CombiHIVVAC vaccine developed by the FSRI SRC VB "Vector" [1, 2]. TBI protein is generated in *E.coli* procaryotic cells. The level of gene expression of recombinant TBI protein at the stage of biomass is determined the quality of the inoculate, the conditions

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of cultivation and the composition of the nutrient medium. All further stages of isolation and purification of recombinant TBI protein accumulated in inclusion bodies depend on the yield of cells. Thus, when developing cultivation conditions, it is necessary to find an optimal combination of parameters that provide a stable and high yield of the target protein.

The goal of the study is to optimize the cultivation process that ensures the yield of biomass with a high content of TBI protein.

## MATERIALS AND METHODS

Recombinant *E.coli* JM 103/pTBI producing strain was used for optimization of the cultivation process.

Experimental multicomponent TB and TB plus media were used as nutrient media. YT medium served as a control [3, 4, 9]. TB medium consisted of tryptone, 12.0 g/l; yeast extract, 12.0 g/l; glycerol, 4.0 g/l; $KH_2PO_4$, 2.31 g/l and $K_2HPO_4$, 12.5 g/l, ampicillin, 200 μg/ml. TB plus medium was supplemented with a trace element solution.

Cultivation of *E.coli* JM 103/pTBI recombinant strain was carried out in 750 ml Erlenmeyer flasks containing 100 ml of the nutrient medium. The content of the flasks was shaken at 180 rpm in a New Brunswick E-25 incubator.

The dose of inoculate was 1 % of the nutrient medium volume. In the process of cultivation, nalidixic acid was added as a protein biosynthesis inducer. Upon completion of the fermentation process, biomass was separated by centrifugation (8000 rpm/min, 15 min).

Optical density was measured by the spectrophotometer at 550 nm. The content of recombinant TBI protein in biomass was analyzed by electrophoresis in 12 % PAAG followed by densitometric scanning [3, 5].

Fermentation was conducted in a laboratory fermenter with a working volume of 10 liters (“Ultraferm”, LKB, Sweden) equipped with the systems of measurement of temperature, stirrer speed and aeration rate. The dose of the inoculate was 5 % of the nutrient medium volume and fermentation was carried out for 6-7 h.

Fermentation conditions: 37 °C, 200 rpm/min. Aeration conditions: air was passed at a rate of 1 vvm (volume of air per unit volume of medium per minute) from the beginning of fermentation to the addition of the inducer. Then the air flow rate was increased up to 1.7 vvm.

Isolation and purification of recombinant TBI protein was carried out as follows: the cells were resuspended with buffer (10 mM Tris/HCl, pH 8.0, 1 : 4 v/v) and degraded by ultrasound.

ДЕАЕ-cellulose ДЕ-52 (Whatman, England) was used for chromatographic purification of TBI protein.

SDS/PAGE electrophoresis was performed with 12 % (w/v) gel by the Laemmli method [6].

Quantitative analyses were carried out using a computer program «Gel-pro analyzer, Ver. 3.1» (Media Cybernetics Inc. США). The yield of TBI protein was calculated in percents from the initial content of the total protein.

The content of bacterial endotoxin in the purified protein was evaluated by the LAL test.

## RESULTS

The process of protein biosynthesis in procaryotic cells and the amount of the target product are largely determined by cultivation conditions which should provide effective expression of the cloned gene: realization of genetic and phenotypic properties of the population of recombinant cells (identity and stability of plasmid, bacterial cell phenotype). When cultivating *E.coli* JM 103/pTBI strain in YT medium, the effectiveness of expression was low and the content of TBI protein in biomass was 5-8 % of the total protein. In order to increase the yield of recombinant protein we optimized cultivation conditions of the producing strain by varying the composition of the nutrient medium, the time of addition of nalidixic acid and the aeration rate. At the first stage the process of microbiological synthesis was carried out in flasks. TB medium with or without trace elements was chosen as an experimental medium for cultivation of recombinant *E.coli* JM 103/p TBI strain. YT medium served as a control. TB and TB plus media differ from YT medium by the multicomponent composition and the presence of the additional source of carbon – glycerol [7-9].

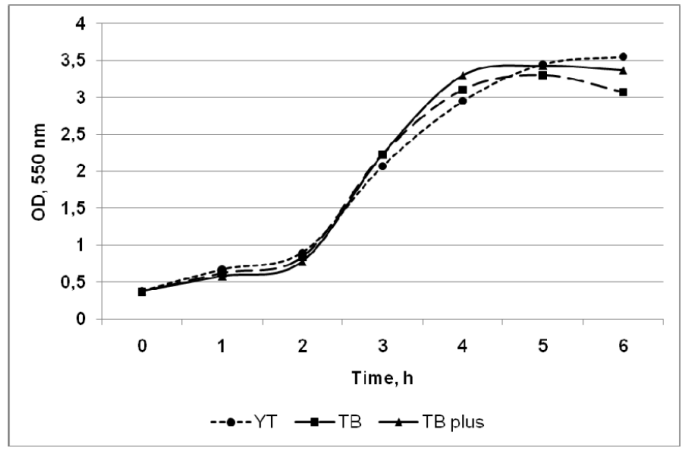


Figure 1. The growth curves of *E.coli* JM 103/pTBI in optimized media.

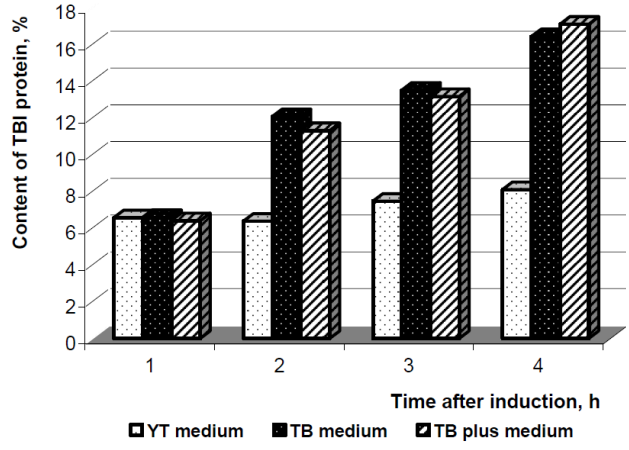


Figure 2. The dynamics of accumulation of TBI protein in the process of cultivation of *E.coli* JM 103/pTBI in optimized media.

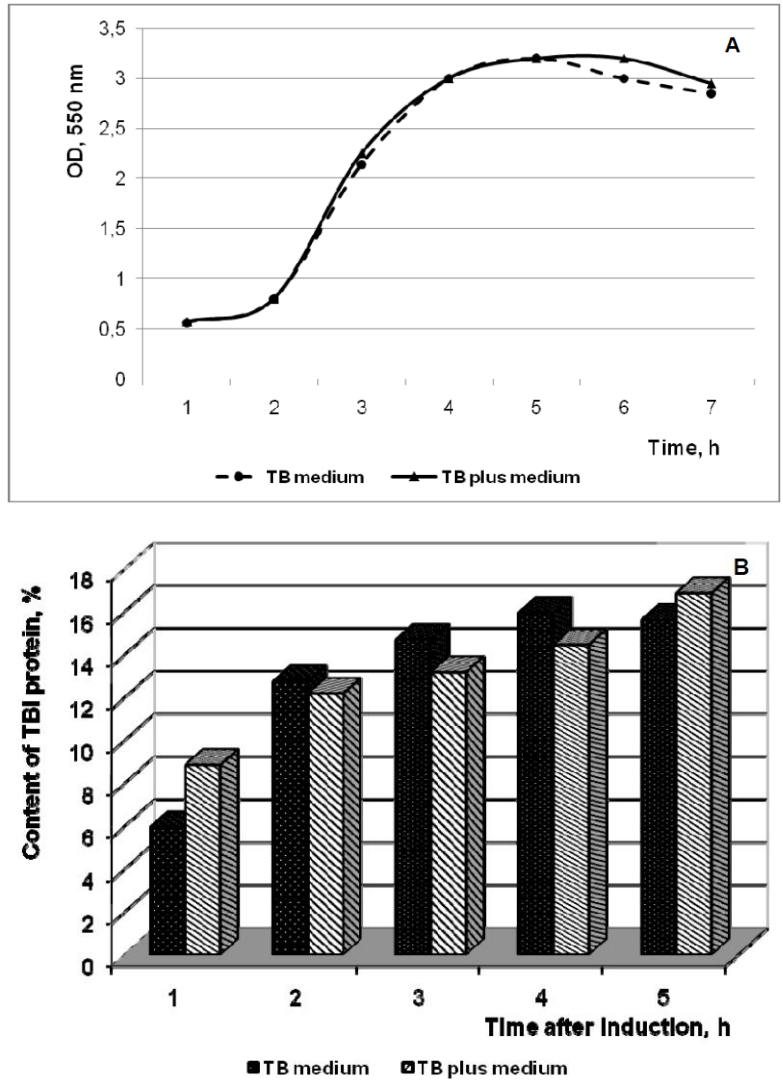


Figure 3. The growth curves of *E.coli* JM 103/pTBI and accumulation of TBI protein in optimized media after induction at an optical density of 0.8. A – Growth curves. B – The dynamics of accumulation of TBI protein.

The process of cultivation was carried out as described in Materials and Methods. The culture was grown up to an optical density of 0.7-0.8, then nalidixic acid was added to a final concentration of 90 μg/ml. Optical density was measured every hour. Following induction the content of recombinant TBI protein was determined.

The data obtained are presented in Figures 1, 2. It was demonstrated that despite the absence of differences in cell concentration the content of recombinant TBI protein in optimized media was significantly higher as compared to the control. The amount of TBI protein was 15.8 ± 0.5 % in TB medium and 16.2 ± 0.4 % in TB plus medium versus 8.1 ± 0.4 % in YT medium.

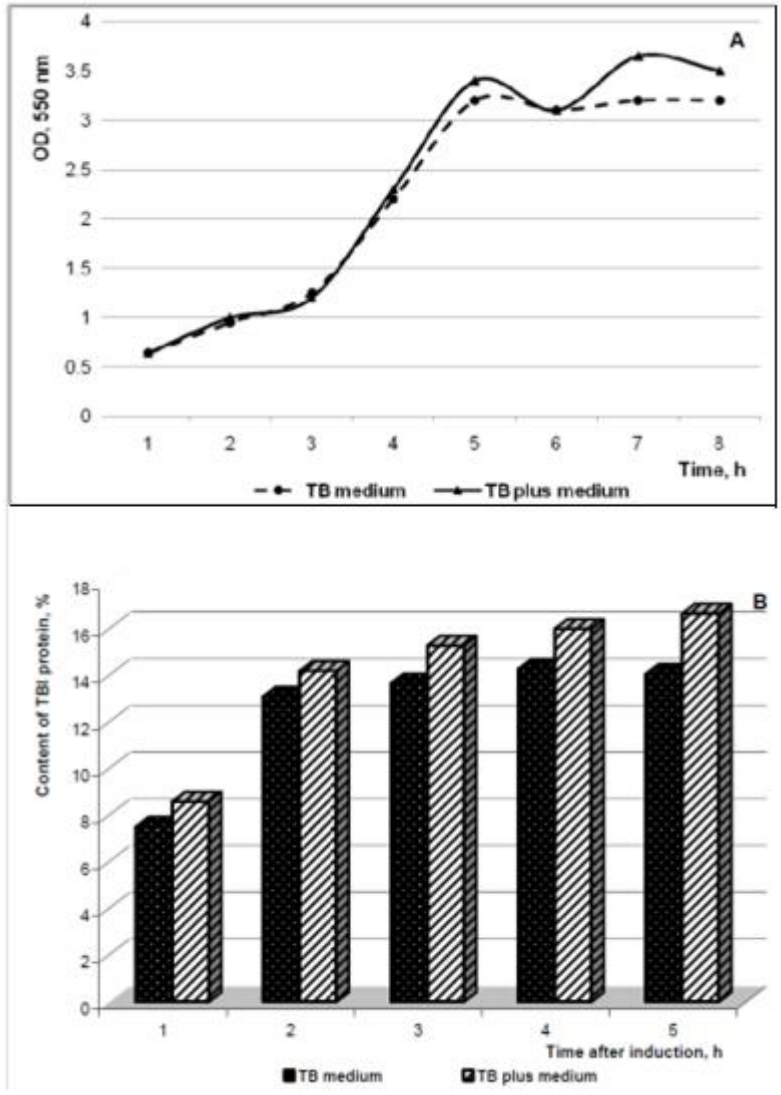


Figure 4. The growth curves of *E.coli* JM 103/pTBI and accumulation of TBI protein in optimized media after induction at an optical density of 1.2. A – Growth curves. B – The dynamics of accumulation of TBI protein.

At the second stage the dynamics of accumulation of the target protein was studied depending of the time of addition of nalidixic acid. Cultivation was carried out in Erlenmeyer flasks using optimized TB and TB plus media. The culture was grown to an optical density of 0.8, 1.2 and 1.5. Then nalidixic acid was added to a final concentration of 90 μg/ml. Optical density was measured every hour. Following induction the content of recombinant TBI protein was determined. The data obtained are presented in Figures 3, 4, 5.

It is seen that at the end of cultivation the concentration of cells did not depend on the composition of the medium and the time of addition of the inducer. The protein content in TB plus medium was higher than in TB medium. The maximal content of TBI protein constituted 15.6 % in TB medium in the case of addition of the inducer at an optical density of 0.8. The

content of the target protein in TB plus medium did not depend on the time of addition of nalidixic acid and, on the average, was 16.5 ± 0.4 % of total cellular proteins. At the third stage the cultivation process of *E.coli* JM 103/pTBI was transfered from shaken flasks to a laboratory fermenter. The parameters of fermentation were chosen in accordance with the results of previous experiments that allowed us to enhance the content of the target protein. TB plus media was chosen as a cultivation medium, TB medium served as a control. Nalidixic acid was added at an optical density of 0.8-1.0. Air was passed as earlier described.

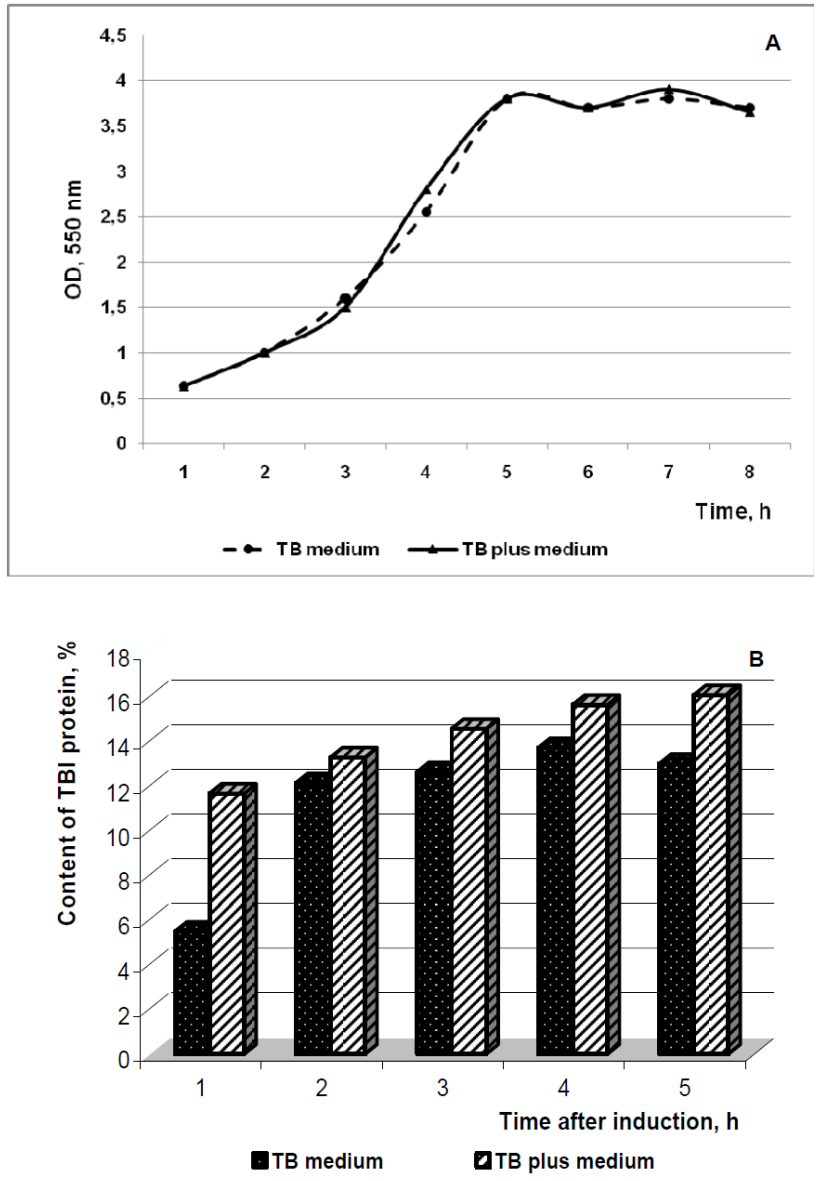


Figure 5. The growth curves of *E.coli* JM 103/pTBI and accumulation of TBI protein in optimized media after induction at an optical density of 1.5. A – Growth curves. B – The dynamics of accumulation of TBI protein.

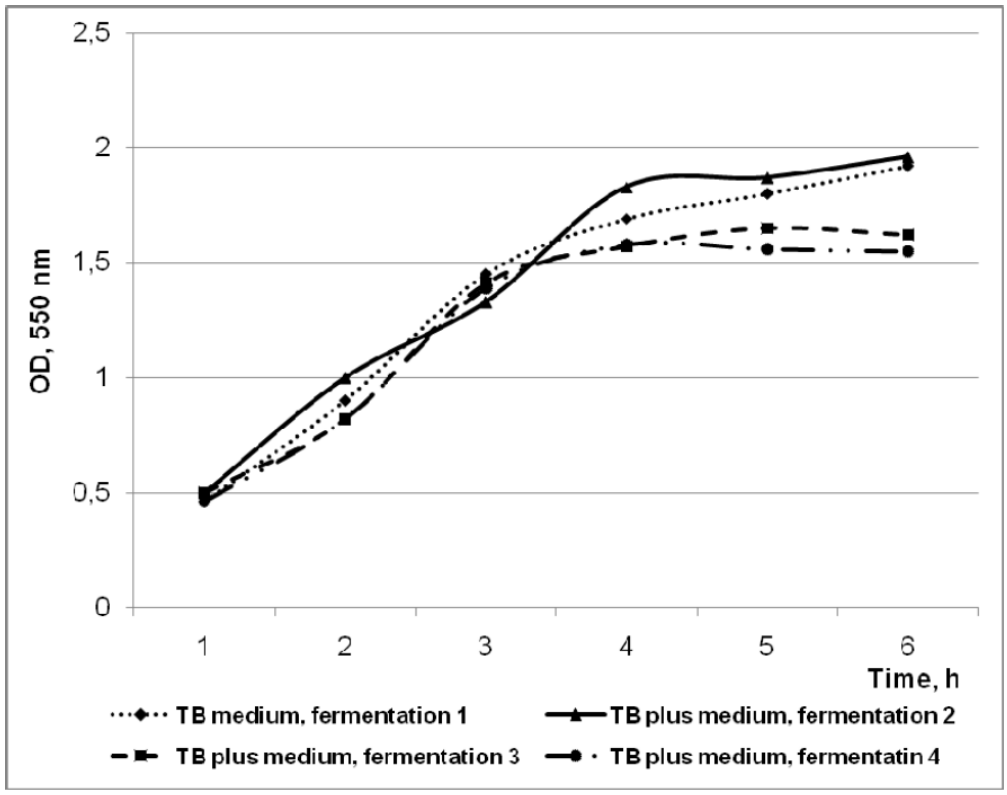


Figure 6. The growth curves of *E.coli* JM 103/pTBI in a laboratory fermenter.

The dynamics of cell concentration and the content of TBI protein were analyzed in the process of fermentation. Three fermentation processes were carried out in TB plus medium and one – in TB medium.

The first fermentation was carried out in TB medium. In this case nalidixic acid was added at an optical density of 0.9. Whereas the yield of cells was 19.6 g the content of the protein in biomass was lower than in flasks and amounted to 13.3 %.

The second fermentation was carried out in TB plus medium. Nalidixic acid was added at an optical density of 1.0.

The yield of cells was 21.8 g and the content of the protein in biomass – 16.4 %, which corresponded to the data obtained during cultivation in flasks. To assess the influence of the time of addition of nalidixic acid on the concentration of cells and the content of protein in biomass the subsequent fermentation processes were carried out with adding nalidixic acid at an optical density of 0.8.

It was shown that the content of protein in biomass amounted to 18.2 % and 17.4 % at practically the same concentration of cells. The yield of cells constituted 15.4 g and 16.0 g, respectively, and was lower in comparison with the second fermentation.

Thus, the content of TBI protein in biomass remained high and did not depend on the time of addition of nalidixic acid in TB plus medium, which corresponded to the data obtained in cultivation flasks.

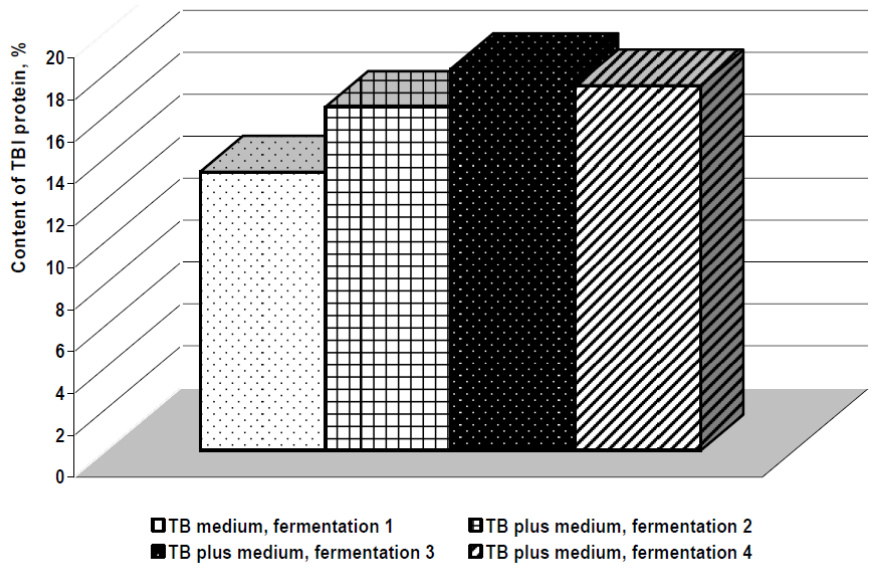


Figure 7. Accumulation of TBI protein in the process of cultivation in a laboratory fermenter.

**Table 1. The comparative characteristics of TBI protein isolated from biomass of recombinant *E.coli JM 103/pTBI* strain**

| Cultivation medium | Electrophoretic purity, % | Yield of TBI protein, % | Pyrogenicity, < 1.4 °C | LAL-test, <25 EU/dose | Molecular weight, kDa |
|---|---|---|---|---|---|
| YT | 92 | 40 | 1.4 | >45 | 20 |
| TB plus | 96 | 60 | 0.5 | <25 | 20 |

Figures 6 and 7 show the growth curves of *E.coli* JM 103/pTBI and the content of the TBI protein in biomass when cultured in a laboratory fermenter.

Finally, isolation of recombinant protein and its chromatographic purification were carried out. Comparative qualitative and quantitative characteristics of TBI protein isolated in the process of cultivation of *E.coli* JM 103/pTBI in TB plus and YT media are demonstrated in Table 1.

It is seen that qualitative and quantitative characteristics of TBI protein isolated in the process of cultivation of *E.coli* JM 103/pTBI in TB plus medium were better than those in YT medium. When TB plus medium was used the yield of the target TBI protein was 60 % of its initial content, with a purity of 96 % as compared to YT medium, in which the yield was 40 %, with a purity of 92 %.

## CONCLUSION

1. The optimal composition of the medium for cultivation of recombinant. *E.coli* JM 103/pTBI strain has been chosen. The content of the target protein was increased in biomass by two-fold as compared to the control. The maximal yield of TBI protein was in TB plus medium.

2. It has been shown during cultivation in flasks that accumulation of TBI protein in TB plus medium did not depend on the time of addition of nalidixic acid at an optical density of 0.8, 1.2 and 1.5 and constituted, on an average, 16.5 ± 0.4 %.
3. When cultured in a laboratory fermenter the maximal content of target protein in biomass reached 18.2 % following induction at an optical density of 0.8.
4. Not only quantitative, but also qualitative characteristics of target TBI protein were improved as a result of optimization of the process of cultivation of recombinant, *E.coli* JM 103/pTBI strain.

## ACKNOWLEDGMENTS

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*Chapter 5*

# OBTAINING AND PROPERTIES OF INTRACELLULAR AMINOACYLASE *ESCHERICHIA COLI*

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## ABSTRACT

Isolation and purification method of intracellular aminoacylase *Escherichia coli* with use of ion-exchange chromatography on DEAE cellulose is developed. Ferment is obtained with 30% yield activity and 32-fold purified. Physico-chemical properties of investigating ferment is studied.

**Keywords:** L-aminoacylase, intracellular ferment, Escherichia coli, chromatography, properties

Aminoacylase (N-acylamino acid amidohydrolase, EC 3.5.1.14) hydrolyzes amide bonds of N-acylated amino acids. This ferment is found in different microorganisms, animals and other objects. The aminoacylase in native as well as in immobilized state is used in industrial scales for production of optically active amino acids. Widely practical application of stereoselective hydrolysis of acylated amino acids became possible due to researches of Greenstein et al. [1], who managed to isolate from racemates practically from all native and some synthetic amino acids with use of homogenate of pig kidney or partially purified preparation acylase I of pig kidney. More detailed investigations of the process of obtaining and study of aminoacylase were performed by Japanese scientists, who used ferment from *Aspergillus oryzae* [2-4] for this purpose. Now there are a lot of articles devoted to obtaining and investigation of properties of different aminoacylase preparations, isolated from different microorganisms: *M. agilis* [5], *Str. olivoreticuli* [6], *Asp. oryzae* [7]. However as opposed to comparatively well studied extracellular aminoacylases, intracellular aminoacylases are not enough characterized. For this reason the study of intracellular aminoacylase is pressing issue.

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The purpose of this work is obtaining (isolation and purification) of intracellular aminoacylase *Escherichia coli* and investigation of it's properties. It was found that in logarithmically growing culture *E. Coli*, possessing aminoacylase activity, intracellular aminoacylase manifests essential fermentative activity in stationar phase of cell growth. Based on this, to isolate the investigating ferment, *E. Coli* cells, having aminoacylase activity were used in stationary phase. Fermentation period was 20 h at 37 °C. Data concerning isolation and purification of intracellular aminoacylase are shown in table 1. Cell extract was obtained by degradation of the cells under ultrasonic desintegrator, followed by centrifugation at 13000 rpm, 20 min, + 4 °C. Precipitation of fractions by nuclear acids was performed by ammonium sulfate at 55-80 % saturation, followed by chromatography on DEAE cellulose at pH 7,0 with a gradient of phosphate buffer concentration from 0,1 M to 0,7 M in the presence of 1mM $CoCl_2$ and 1mM dithiothreitol – managed to escape from most part of ballast proteins. At secondary chromatography active fractions of investigating ferment on DEAE-cellulose at pH 6,0 intracellular aminoacylase *E. coli* is eluted if used sodium chloride gradient concentration from 0,1 to 0,4 M in initial buffer. Active ferment eluted under influence of 0,25 M NaCl on the same buffer (Fig. 1). Based on developed scheme it was possible to obtain intracellular aminoacylase *E. coli* with 30% yield activity and 32-fold purified.

Intracellular aminoacylase *E. coli*, obtained with above-mentioned method was used to study it's physico-chemical properties. The isolated ferment is stable upon storage in 0.1 M phosphate buffer at pH 7,0 in the presence of 1mM $CoCl_2$ and 1mM dithiothreitol at 4°C for several months. pH of the ferment optimal activity by hydrolysis of N-acetyl-D, L-methionine makes up 7,0. The optimum value for the ferment stability is the pH interval 6,0-7,5. In the presence of $Co^{2+}$ ions, the intracellular aminoacylase *E. coli* is rather thermostable, it doesn't lose its activity at 50°C for 60 min. However, at as high temperature as 60°C, it's total inactivation occurs within 30 min. The temperature optimum of the ferment in the presence of 1mM $CoCl_2$ is observed at 37°C.

The total inactivation of intracellular aminoacylase *E. coli* with p-chloromercuri-benzoate indicates availability of free SH-groups that are essential for activity. The isolated ferment is inhibited with ethylenediaminotetraacetic acid that proves metal-dependence of intracellular aminoacylase *E. coli*. Studies of the effect of metal ions on the ferment have shown that the ions of $Co^{2+}$ provide maximum activating action, activating intracellular aminoacylase *E. coli* for 100%, ions of $Ca^{2+}$, $Mn^{2+}$ - is two times less, and the action of other investigated ions of metals ($Cd^{2+}$, $Pb^{2+}$, $Sn^{2+}$) on the ferment activity is ineffective.

Thereby it can be concluded that isolation and purification method of intracellular aminoacylase *E. coli* is developed, which allows to obtain the ferment with 30% yield activity and 32-fold purified. Physico-chemical properties of investigated ferment are studied.

## MATERIALS AND METHODS

Strain of *Escherichia coli LGE 36*, containing recombination plasmid with *arg E* gene, coding aminoacylase synthesis was used in this work (All-Russian collection of industrial microorganisms, "Institute of Genetics and Selection of Industrial Microorganisms", Moscow). To maintain and reproduce the strain, medium with following composition was

used: g/l: $KH_2PO_4$ – 3; $Na_2HPO_4$ – 6; NaCl – 0,5; $NH_4Cl$ – 1; agar – 0,2; $MgSO_4$ – 0,25, tiaminechloride – 0,01; glucose – 6; Na succinate – 6; ampicillin – 0,1; pH medium 7,0±1.

N-acetyl-D, L-methionine, dithiothreitol, Na succinate, ortophtalic aldehyde produced in Serva (Germany), DEAE cellulose (Pharmacia, Sweden), reagents produced in FSU countries were used as well.

Aminoacylase activity was determined by method [8]. Reaction mixture contained: 0,045 M N-acetyl-D, L-methionine; 0,05 M Na, K-phosphate buffer, pH 7,0; 0,2 mM $CoCl_2$, 1mM dithiothreitol and 2,5 μg protein of ferment solution. The samples were incubated at 37°C for 5 min. The reaction was stopped by adding trichloroacetic acid (final concentration – 6%). The liberated L-methionine was determined by adding 1,25 ml distilled water and 1,25 ml reagent of ortophtalic aldehyde (60 ml 0,1M borate buffer, pH 9,7; 10mg of ortophtalic aldehyde; 0,005 ml of mercaptoethanol) to 0,05 ml of sample, followed by measurement of optical density of obtained solutions at wavelength 340 nm. As a unit of acylase activity is accepted ferment amount catalyzing 1 μmol L-methionine for 1h at above-mentioned conditions. The concentration of proteins was determined by absorption at 280 nm.

**Table 1. Isolation and purification of intracellular aminoacylase *E. coli***

| № | Purification stage | Protein, mg | Total activation, U x$10^3$ | Specific activity, U/mg | Total yield, % | Purification, fold |
|---|---|---|---|---|---|---|
| 1 | Cell extract | 1100 | 2200 | 2000 | 100 | 1 |
| 2 | Precipitation with streptomycine sulfate | 1000 | 2100 | 2100 | 95 | 1,05 |
| 3 | Fractioning with ammonium sulfate | 160 | 1680 | 10500 | 80 | 5 |
| 4 | Chromatography on DEAE cellulose, at pH 7,0 | 25,8 | 814 | 31500 | 37 | 16 |
| 5 | Chromatography on DEAE cellulose at pH 6,0 | 10,5 | 660 | 63000 | 30 | 32 |

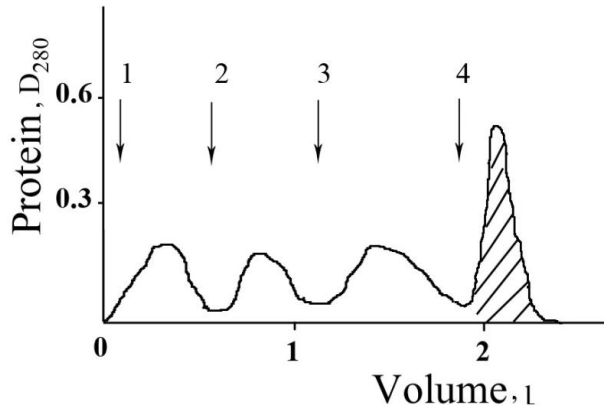


Figure 1. Chromatography of intracellular aminoacylase *E. coli* on DEAE cellulose, at pH 6,0. Stepwise natrium chloride gradient is shown with arrows: 1 - 0,1 M NaCl; 2 – 0.15 M NaCl; 3 – 0,2 M NaCl; 4 – 0.25 M NaCl. Active fractions of the ferment is shadowed.

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*Chapter 6*

# DEVELOPMENT OF HRP-FUNCTIONALIZED CARBON-COATED IRON NANOPARTICLES USING ARENEDIAZONIUM TOSYLATES

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## ABSTRACT

The present article describes the method of immobilization of biomolecules (Horseradish peroxidase) on carbon-coated iron nanoparticles using arenediazonium tosylates. As a result, a magnetically controlled complex of biomolecules and nanoparticles was created. The complex formation was confirmed by FT-IR spectroscopy. It has been shown that peroxidase attached to nanoparticles preserves its enzymatic activity.

**Keywords:** Carbon-coated nanoparticles, diazonium salt, surface modification, HRP, immobilization, enzymatic activity

## INTRODUCTION

The constructions based on superparamagnetic nanoparticles, which are covalently bound with protein molecules (such as enzymes, antigens, antibodies), are particularly useful in biomedical diagnostics and environmental monitoring [1]. Due to their small size and high surface area, these constructions effectively interact with the target molecule directly as well

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as significantly increase sensitivity of analysis. Due to superparamagnetic properties of nanoparticles it is possible to control movements of these constructions and detect them when the external magnetic field overlaps [2, 3]. However, creation of constructions based on nanoparticles requires development of effective methods for immobilization of biomolecules which will not lead to disturbance of their structure and function. It has been shown that aryldiazonium chlorides $ArN_2^+$ $Cl^-$ interact with the surface of certain carbon-coated nanoparticles in the presence of nitrogen molecules; as a result of this the aryl (Ar) radical bonded with the surface [4]. However, these reagents were characterized by a series of significant disadvantages related to their low stability and explosion hazard and required special reaction environment. Relatively stable diazonium salts (aryldiazonium tetrafluoroborates $ArN_2^+$ $BF_4^-$) are known, however, their application is restricted by their low stability in water and organic solvents. Recently we have obtained a new type of stable diazonium salts: aryldiazonium tosylates $ArN_2^+OTs^-$ (ADT) [5]. ADTs are characterized by higher applicability in comparison with the known diazonium salts: stability in water and organic solvents, high storage stability, and explosion safety (confirmed by DSC data). We demonstrated the possibilities of ADT to modify the surface of carbon-coated iron nanoparticles [6, 7]. In this study, we investigated enzymatic activity of horseradish peroxidise (HRP) immobilized onto carbon-coated iron nanoparticles, using arenediazonium tosylates.

## MATERIALS AND METHODS

The carbon-coated iron nanoparticles were used as a core of the developed construction. The Fe@C nanoparticles (7-10 nm) were obtained by gassphase synthesis [6]. The 4-carboxybenzenediazonium tosylates were obtained according to the described procedures [5]. The surface of nanoparticles was modified by 4-carboxybenzenediazonium tosylate [7]. The method of surface functionalization and biomolecule immobilisation is shown on Figure 1.

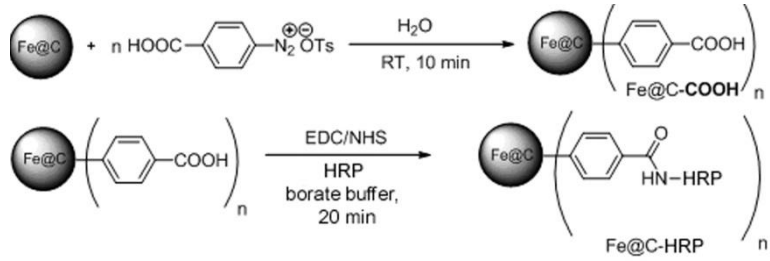


Figure 1. Scheme of the method of HRP immobilisation onto carbon-coated iron nanoparticles.

Horseradish peroxidase (HRP) (Merck, Germany) was diluted in water to the final concentration $2.8*10^{-5}$ mol/L. The immobilization was carried out in a borate buffer (pH 8.6) containing $0.84*10^{-5}$ mol/L of HRP and 3 mg of the modified Fe@C nanoparticles. For coupling of HRP with COOH-groups on the surface the EDC/NHS chemistry was applied. The coupling procedure was carried out according to the protocol [8]. After immobilisation the obtained complex was separated from the solution by magnetic separation using a permanent magnet (0.2 T) and washed consecutively with the borate buffer, twice with $H_2O$

and PBS buffer (pH 7.4). Then the HPR-Fe@C complex was diluted in the PBS buffer. The amount of HRP bound to nanoparticles was determined spectrophotometrically by solution depletion (Unico 2800, United Product and Instrument INC, United States). The concentration of HRP was determined using extinction coefficient $E_{403} = 95000\ M^{-1}\ cm^{-1}$ [9].

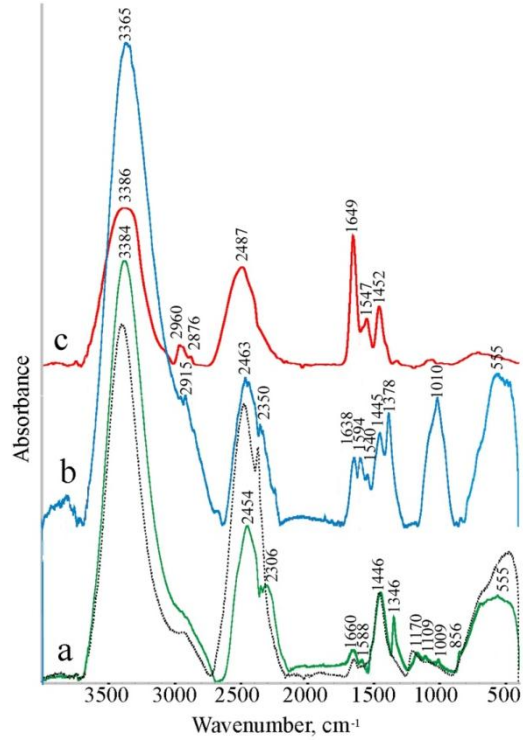


Figure 2. ATR-IR spectra of carbon coated iron nanoparticles (dashes black line), ADT-modified carbon coated iron nanoparticles (green line a), HRP immobilized onto carbon-coated iron nanoparticles (blue line b) and free HRP (red line c).

HRP activity was measured using the solution containing 0.2 mM of o-phenylenediamine (OPD) as a substrate, 17 mM of $H_2O_2$ in a 20 mM sodium-citrate buffer (pH 5.0) [10]. The HRP solution in the PBS buffer was used as control. The initial velocity ($V_0$) of the reaction was calculated using the method of numerical differentiation at the initial point of a kinetic curve. Statistical analysis was performed using Statistica 6.0.

The $D_2O$ solution infrared spectra were recorded on IR-Fourier spectrometer Nicolet 6700 by the ATR method on the diamond crystal in the range of 4000-400 $cm^{-1}$ by 128 scans for each sample with the resolution of 4 $cm^{-1}$.

## RESULTS AND DISCUSSIONS

Spectrophotometric analysis has shown that binding capacity of HRP with nanoparticles constituted $0.49*10^{-6}$ Mol per gram.

The protein-nanoparticle complex formation was confirmed by FT-IR spectroscopy (Figure 2). In ATR-IR-spectra of the complexes characteristic protein adsorption bands

appeared in the range 1700-1600 $cm^{-1}$ (amide I), caused by C=O stretching vibrations of peptide linkages, and 1600-1500 $cm^{-1}$ (amide II), resulted from a combination of N–H in plane bending and C–N stretching vibrations of the peptide groups. The broad adsorption band at 550 $cm^{-1}$ belongs to Fe@C nanoparticles [4]. After ADT-modification in ATR-IR spectra of nanoparticles the characteristic adsorption bands of benzene ring vibration in range 500-1100 $cm^{-1}$ and COOH group vibration at 1346 $cm^{-1}$ are appeared [4, 11].

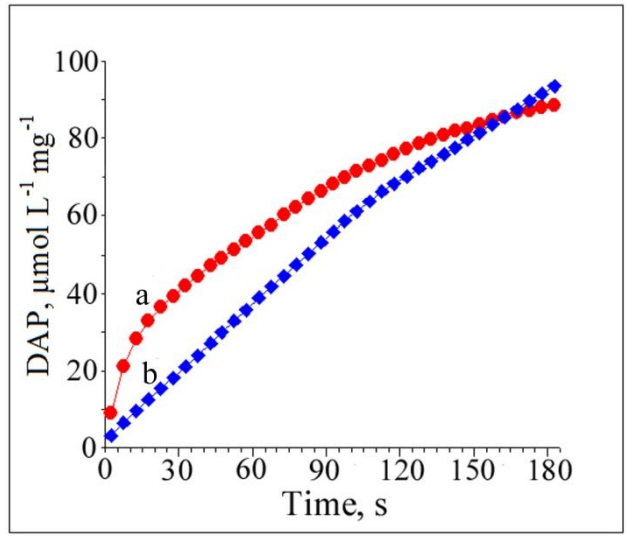


Figure 3. DAP (2,3-diaminophenazine) formation in the presence of $H_2O_2$ as a function of time. The reaction is catalyzed by free HRP (red line 1) and HRP immobilized onto carbon-coated iron nanoparticles (blue line 2).

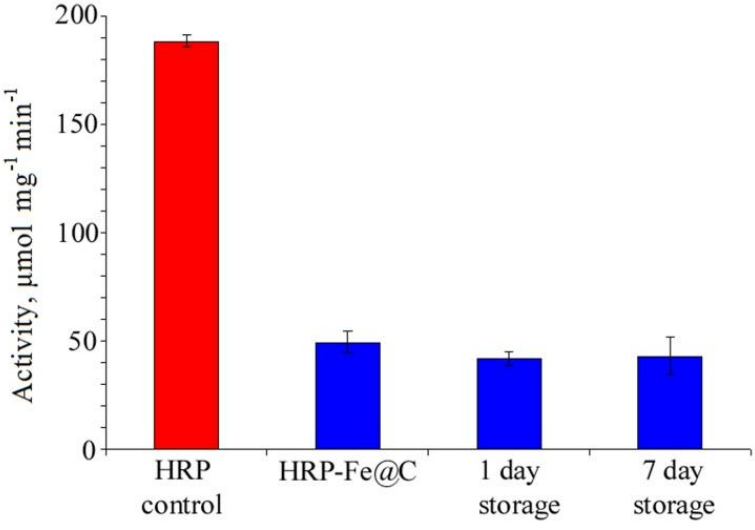


Figure 4. Enzymatic activity of free (control) HRP (red column) and HRP immobilized onto carbon-coated iron nanoparticlesafter preparation (blue column) and after storing 1 and 7 days.

The HRP-Fe@C complex formation is associated with changes in IR spectra of both nanoparticles and HRP. The observed increase in the intensity of vibrations at 1010 $cm^{-1}$ is caused by stretching of the ADT C-C bond on the nanoparticles surface when binding with peroxidase. The shift of the adsorption band of the COOH group on the modified nanoparticles surface from 1346 to 1377 $cm^{-1}$ after HRP immobilization is the result of bonding of the carboxyl group with HRP. The shift of the adsorption bands amide I from 1649 to 1638 $cm^{-1}$ and amide II from 1547 to 1540 $cm^{-1}$ in HRP-Fe@C IR spectra is caused by HRP immobilization on the nanoparticle surface [12].

Amide I and amide II adsorption bands are the most useful markers for conformation (secondary structure, i.e., a-helix and b-sheet) of the polypeptide backbone chain. These adsorption bands are shifted to higher frequencies under denaturation of the HRP molecule. However, presence of HRP-Fe@C bands in IR spectrum at 1638 $cm^{-1}$ and cm 1540 $cm^{-1}$ allows concluding immobilization does not alter the spatial structure of HRP or damage HRP leading to a denatured state. [13]. It has been determined that HRP attached to nanoparticles, can catalyze the oxidation of o-phenylenediamine in the presence of $H_2O_2$. (Figure 3). The enzymatic activity of free and nanoparticle-attached HRP was 188,5±2.5 and 49,5±4.9 $\mu mol*min^{-1}*mg^{-1}$ respectively.

The enzymatic activity of HRP-Fe@C has not significantly decreased for at least 7 days, when stored at RT, and constituted 43.1±8.5 $\mu mol*min^{-1}*mg^{-1}$ (Figure 4).

## CONCLUSION

We introduce a new effective method of superparamagnetic nanoparticle functionalization. Immobilization does not lead to disturbance of functional activity and conformation of the protein molecule.This new method of magnetically controlled construction formation, in which proteins are covalently attached to nanoparticles, have a great applicability for biotechnology and biomedicine.

## ACKNOWLEDGMENTS

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*Chapter 7*

# EXPERIMENTAL APPROACH TO THE INDUCTION OF NONCULTURABLE STATE OF *LACTOCOCCUS LACTIS*

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## ABSTRACT

Long-term exposure of microbial populations to physical, chemical and biological stresses and their combinations causes formation of viable but nonculturable (VBNC) cells. Detection of VBNC in the environment, food, and living organisms is a problem of high medical and biological significance.

We have studied 3 bacteriocin producing strains of *Lactococcus lactis* ssp. *lactis* (194, 729 and MGU). Obtained data show that the examined strains are capable of entry into VBNC state within 2 weeks under carbohydrate starvation stress. However, synthesis of antimicrobial compounds (bacteriocins) remained unaltered.

**Keywords:** Nonculturable, Lactococcus lactis, VBNC, carbohydrate starvation

## INTRODUCTION

Long-term exposure of microbial populations to physical, chemical biological stresses and their combinations causes formation of viable but nonculturable (VBNC) cells. These cells lose the ability to reproduce and slow down their metabolism to ensure survival of the population through the stress period. VBNC state is known for a wide variety of microorganisms. These include members of *Escherichia*, *Vibrio*, *Klebsiella*, *Salmonella*, *Shigella*, *Legionella*, *Pseudomonas*, *Lactococcus* and many other genera (Byrd J.J. et al.,

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1991; Rahman I et al., 1996; Steinert M. et al., 1997; Gupte A.R. et al 2003; Bates T.C., Oliver J.D., 2004; Eschbach M. et al., 2004; Ganesan B. et al, 2007; Sachidanandham R., et al. 2009). Possible presence of VBNC cells in environmental, clinical, food and other samples makes results obtained with the use of only traditional culture techniques unreliable. *Lactococcus lactis* can enter VBNC state in stored cultured milk foods such as ageing cheeses.

Detection of VBNC lactococci in the environment, milk products, organs of warm-blooded organisms and biomedical preparations is of great microecological and biomedical significance.

## MATERIALS AND METHODS

For this study we chose 3 bacteriocin producing strains of *Lactococcus lactis* subsp. *lactis* (194, 729 and MGU). To induce VBNC state we used a synthetic medium that creates carbohydrate starvation conditions, suggested by Ganesan B. et al, (2007). Formation of VBNC cells was confirmed by difference between numbers of colony forming units (CFU/ml) and total number of viable and dead cells. Percentage of viable cells was determined in fluorescence microscope by their esterase activity after staining with Live/Dead double cell staining kit, Fluka. Total counts were made in a Goryaev counting chamber. CFU/ml value was determined by plate counts on the following medium (g/l): sucrose – 20, $KH_2PO_4$ – 20, NaCl – 2, $MgSO_4$ – 0.2, yeast extract – 20, agar-agar – 15, pH 7.0.

## RESULTS AND DISCUSSION

Data on viability of *L. lactis*, obtained during six weeks of incubation in starved medium are shown in the table. Our results show that after two weeks of incubation number of CFU/ml dropped by 3 orders of magnitude ($5x10^4$/ml average) and after 6 weeks – by 3–4 orders of magnitude ($0,52 – 7,4x10^4$). Total viable counts remained fairly constant ($3,4x10^7$/ml average after 2 weeks and $6,79x10^7$ after 6 weeks).

Culturable part of the population exhibited slowed growth rates: colonies reached maximum size only by 48 hours. We found strain dependent differences in the rate of slowing of colony growth speed. They were determined by comparing colony sizes after 24 hours of culturing and times of their appearing (24-96 hrs.). At the beginning colonies reached 2-3 mm in size within 24 hrs., after 2 weeks – 1–2 mm, and after 3 weeks colony size was less than 1 mm. Smallest colonies (after 24 hrs.) were observed for the strain 194. Their size varied from barely visible to 0,5 mm. However upon the 4-th day the highest number of new colonies (compared to the second day) was seen for strain MGU – up to 50% of total number.

Strain 729 showed phenotypic variability (emergence of transparent colorless colonies with rough edges and less amounts of white exopolysaccharides). By the 5-th week their numbers amounted to 93,6% (figure 1). This may be due to slowing of some metabolic pathways in cells that are stored in carbohydrate starvation conditions for long periods. For strain MGU in some cases we observed small colonies growing at the borders of bigger colonies (figure 2). These colonies appeared several days after the big one reached its

maximum size (no separate colonies that appeared at the same time were observed on these plates). It is possible that big colony produces some compounds that resuscitate some of nonculturable cells around it. Strain 194 exhibited only white round colonies typical for *L. lactis* (figure 3). Biosynthetic activity for antimicrobial compounds (bacteriocins) remained unaltered. After 6 weeks of incubation number of CFU/ml increased four times. This was preceded by significant more than one order of magnitude increase in numbers of dead cells in the culture a week before. It is possible that some components of dead cells promoted resuscitation of some VBNC cells that are the easiest to regain the ability to proliferate. These data conform to published information on induction of nonculturable state in *L. lactis* (Ganesan B. et al, 2007).

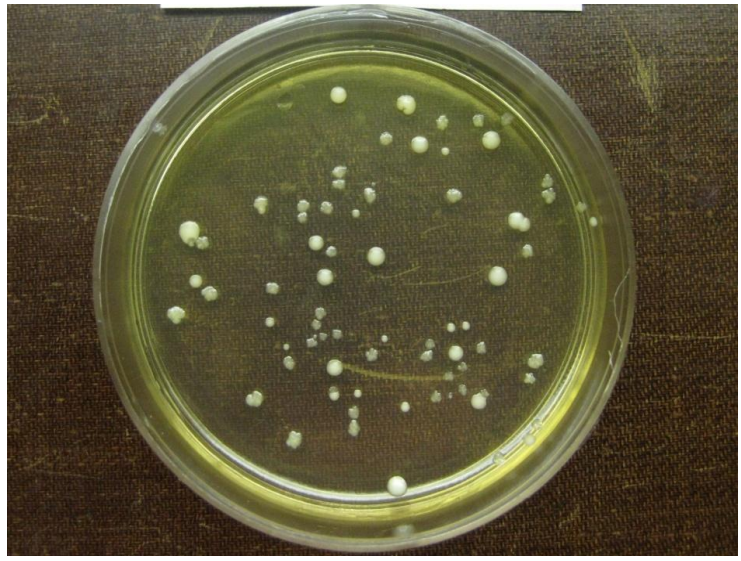

Figure 1. Colony morphology of *Lactococcus lactis* ssp. *lactis* strain 729.

Figure 2. Colony morphology of *Lactococcus lactis* ssp. *lactis* strain MGU.

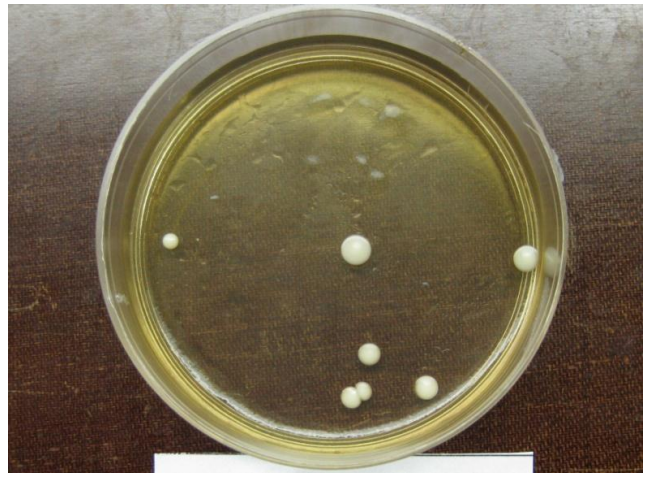

Figure 3. Colony morphology of *Lactococcus lactis* ssp. *lactis* strain 194.

**Table. Viability patterns for different strains of *Lactococcus lactis***

| Period of starvation | *Lactococcus lactis* MGU | | *Lactococcus lactis* 194 | | *Lactococcus lactis* 729 | |
|---|---|---|---|---|---|---|
| | CFU/ml | Viable cells/ml with Live/Dead | CFU/ml | Viable cells/ml with Live/Dead | CFU/ml | Viable cells/ml with Live/Dead |
| 0 weeks. | $(1,33\pm0,14)\times10^7$ | $(1,05\pm0,12)\times10^7$ | $(4,33\pm0,47)\times10^7$ | $(1,52\pm0,17)\times10^7$ | $(2,1\pm0,23)\times10^7$ | $(1,25\pm0,13)\times10^7$ |
| 2 weeks. | $(2,78\pm0,3)\times10^4$ | $(3,40\pm0,37)\times10^7$ | $(5,50\pm0,61)\times10^4$ | $(3,45\pm0,38)\times10^7$ | $(3,00\pm0,33)\times10^4$ | $(3,29\pm0,36)\times10^7$ |
| 5 weeks. | $(5,6\pm0,61)\times10^3$ | $(3,23\pm0,35)\times10^7$ | $(3,87\pm0,4)\times10^4$ | $(3,22\pm0,35)\times10^7$ | $(1,85\pm0,2)\times10^4$ | $(3,69\pm0,41)\times10^7$ |
| 6 weeks. | $(5,20\pm0,57)\times10^3$ | $(4,59\pm0,5)\times10^7$ | $(1,8\pm0,2)\times10^4$ | $(4,47\pm0,49)\times10^7$ | $(7,4\pm0,81)\times10^4$ | $(6,79\pm0,74)\times10^7$ |

## CONCLUSION

We studied viability patterns of 3 strains of *Lactococcus lactis* using different methods.

Formation of VBNC cells of lactococci was registered after 2 weeks when cells were incubated in carbohydrate starvation conditions.

Ability to form VBNC cells and speed of culturability loss are strain dependent.

Obtained data can be used to assess numbers of viable lacococcus cells in environmental and food samples and in biomedical preparations.

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*Chapter 8*

# CALLUS CULTURE TECHNOLOGY OF SPRING SOFT WHEAT STRESS TOLERANT VARIETIES SELECTION

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## ABSTRACT

Spring soft wheat varieties *in vitro* selection for drought, salinity and acidity tolerance technology for Siberian local breeding material was developed. Multiple regeneration phenomenon of wheat callus culture was investigated for the first time. Method of cereal crops test of acidity tolerance was improved.

**Keywords**: Triticum aestivum, in vitro, drought, salinity, low pH, tolerance, test methods

## INTRODUCTION

Earth population is growing and increase of food supply is demanded. New territories which development is impossible without new high-adaptive crop varieties creation are involved in crop rotation. Soil salinity, acidity and drought become the factors which limit crop yield. Classic methods of selection often can`t solve this problem in a required short space of time [[1]]. Genetically modified plants are forbidden to be grown in most part of developed countries in addition.

So far callus culture *in vitro* can become effective ecology safe technology of high stress tolerant wheat varieties creation. This method are widely used in crop selection [[2]], especially wheat one [[3], [3], [5]]. Technology of spring soft wheat varieties selection for drought, soil acidity and salinity tolerance at the base of local Siberian breading material was developed.

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## MATERIALS AND METHODS

12 varieties of spring soft wheat, differed in adaptive rate and vegetation period, and 16 regenerants, which had been created on selective (salt (RS), acidity (RA) and control (RC) media, were involved in research. Isolated plant tissues culture methods [[6]], especially barley tissue ones [[7]] were used for technology development and stress-tolerant regenerants creation processes. Selection was divided into three steps: callus induction, its proliferation and plant regeneration. Immature embryos taken at 20-21 or 27-28 days after the beginning of the earing were used as explants. Cultivation was undertaken on modified Murashige-Skoog medium (MS). 2,4-dichlorphenoxyacetic acid (2,4-D) was used as callus formation inductor. Its level in induction and proliferation media had being optimized while investigation. Expanded calluses were passaged to regeneration media, containing different concentrations of IAA and kinetin or BAP, from proliferation one. Selective conditions, low acidity, high concentration of NaCl for salinity simulation, of polyethylene glycol for drought simulation, were created for tolerant cell lines selection at the two last steps. MS medium (pH 5.6-5.7) was used as control. Levels of hormones and stress agents are described further. Laboratory test of regenerants and their parental varieties salt tolerance was realized by roll method [[8]]. Weight and length of plantlet organs in salt solution in comparison with clear water were measured. Acid tolerance was tested by modified method using coefficient of tested plants organ growth function reduction, which development is described further.

## RESULTS

### Hormone Media Composition Optimization and Detection of Explants Parameters Suitable for Technology

There are great variety of *in vitro* stress-tolerant wheat and barley selection protocols, described in literature and differed in medium composition and cultivation conditions, because of genotype great influence on callus culture growth and development [[9], [10], [2], [7]]. Different levels (0,5; 1; 2; 3 mg/l) of 2,4-D was used for callus induction. 100% level of callus formation was detected on the medium with 3 mg/l 2,4-D. But no regenerants formed at the further steps of selection process after cultivation under these conditions. Calluses were passaged to proliferation medium with halved 2,4-D level in comparison with induction medium after 30 days of cultivation. The most active callus proliferation was detected on the medium contained 0,5 mg/l 2,4-D. So far big calluses could be divided into parts to increase the quantity of samples.

As the result the 2,4-D concentration of 1 and 0,5 mg/l in induction and proliferation medium respectively were chosen for further research in spite of the fact that maximum level of callusogenesis was detected at the 0,25 mg/l of 2,4-D (Table 1). But our latest investigations cleared out that the most suitable for maintenance regeneration activity during further cultivation is proliferation medium with 2.4-D level the same as in callus induction one – 1 mg/l (Figure 1). Vegetative period of parental varieties also had a great influence on callus induction. Early cultivars (Tajozhnaja, Novosibirskaja 15) had significant larger percentage of formatted calluses, than late cultivars (Minusa, KS-1607) independently of 2.4-

D level. They had equal proliferation activity at the second cultivation step. The most active regenerant formation was detected at early cutivars culture, that are the most called-for under Siberian field conditions.

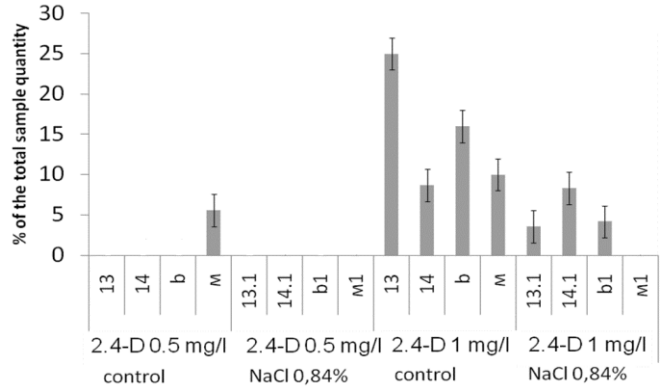


Figure 1. Quantity of regeneration callus passaged from proliferation medium vary in 2.4-D level to the different regeneration medium.

**Table 1. Wheat callus culture growth characteristic (% of the total sample quantity)**

| Genotype | Callus induction medium | | callus proliferation medium | | | | | | | Regenerant yield | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | proliferating calluses | | Morphogenic calluses | | | | | |
| | | | | | | with shoots | | with roots | | | |
| | 2.4-D concentration | samples with callusogenesis | 2.4-D concentration | Control | NaCl, 0,42% | Control | NaCl, 0,42% | Control | NaCl, 0,42% | Control | NaCl, 0,42% |
| Novosi-birskaja 15 | 0,5 | 72,5 | 0,25 | 74,0 | 53,4 | 35,1 | 17,2 | 47,8 | 21,4 | 35,1 | 35,0 |
| Taezhnaya | | 76,5 | | 67,8 | 61,5 | 44,8 | 40,0 | 40,4 | 43,7 | 38,9 | 36,3 |
| Minusa | | 40,8 | | 77,5 | 43,2 | - | 15,9 | 38,4 | 15,9 | - | 15,9 |
| KS-1607 | | 56,7 | | 59,8 | 73,7 | - | 26,7 | - | 26,7 | - | 20,0 |
| Novosi-birskaja 15 | 1 | 72,9 | 0,5 | 92,5* | 78,0* | 24,2* | 23,6 | 20,0* | 23,6 | 24,2* | 25,7* |
| Taezhnaya | | 80,6 | | 72,7 | 70,9* | - | 33,3 | 5,1* | 18,4* | 7,7* | 18,4* |
| Minusa | | 44,9 | | 86,9 | 68,3* | 4,8 | - | 15,9* | - | - | - |
| KS-1607 | | 59,5 | | 76,9* | 54,2* | 27,2 | 8,3* | 27,2 | 8,3* | 27,2 | 8,3* |
| $LSD_{0,05}$ for factor | «genotype» | 13,8 | | 13,7 | | 13,8 | | 15,6 | | 14,1 | |
| | «2.4-D concentration» | 10,8 | | 9,69 | | 9,75 | | 11,0 | | 9,96 | |

*- significant difference induced by 2.4-D concentration change within the genotype (P≤0.05).

**Table 2. Wheat embryos of different age culture growth and development characteristic (% of the total sample quantity)**

| Embryo`s age | Genotype | Callus proliferation medium | | | | Regenerant yield | |
|---|---|---|---|---|---|---|---|
| | | Morphogenic calluses | | | | | |
| | | with shoots | | with roots | | | |
| | | Control | NaCl, 0,42% | Control | NaCl, 0,42% | Control | NaCl, 0,42% |
| 21-22 days | Novosibirskaja 15 | 24,2 | 23,6 | 20,0 | 23,6 | 24,2 | 25,7 |
| | Taezhnaya | - | 33,3 | 5,1 | 18,4 | 7,7 | 18,4 |
| 27-28 days | Novosibirskaja 15 | 26,1 | 21,1 | 11,6* | 11,7* | 20,3 | 11,7* |
| | Taezhnaya | 13,3 | 6,6* | - | - | - | - |

*- significant difference between two embryo`s ages within the genotype (P≤0.05).

**Table 3. Influence of NaCl on growth and development of wheat callus culture**

| Parental genotype | Proliferating calluses, % | | | | Regenerating calluses, % | | | | Regenerant yield, % | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Co | NaCl, % | | | Co | NaCl, % | | | Co | NaCl, % | | |
| | | 0,42 | 0,84 | 1,68 | | 0,42 | 0,84 | 1,68 | | 0,42 | 0,84 | 1,68 |
| Taezhnaya | 83,8 | 92,1 | 64,1*[a] | 56,4*[a] | 43,3 | 50,0 | 25,7*[a] | 10,3*[a] | 32,4 | 42,1 | 18,0*[a] | 0,0*[a] |
| Novosibirs-kaja 15 | 76,0 | 69,2 | 56,0* | 46,2*[a] | 44,0 | 57,7 | 41,7[a] | 26,9*[a] | 36,0 | 38,5 | 12,5*[a] | 0,0*[a] |
| KS-1607 | 84,6 | 69,2 | 53,9* | 45,0 | 46,2 | 61,6* | 69,2* | 8,3*[a] | 38,5 | 53,9* | 15,4*[a] | 8,3*[a] |
| Minusa | 77,3 | 80,5 | 50,0*[a] | 65,1 | 65,9 | 63,4 | 31,0*[a] | 20,9*[a] | 59,1 | 43,9* | 23,8*[a] | 0,0*[a] |
| Average data | 80,4 | 77,8* | 56,0*[a] | 60,7* | 49,8 | 58,2 | 41,9[a] | 16,6*[a] | 41,5 | 44,6 | 17,4*[a] | 2,08*[a] |

Co – control medium without NaCl;

*- significant difference between control and selective media (P≤0,05);

a – significant difference between selective medium and medium with minimum selective level (P≤0,05).

**Table 4. Influence of induced drought on growth and development of wheat callus culture**

| Parental genotype | Proliferating calluses, % | | | Regenerating calluses, % | | | Regenerant yield, % | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | PEG 16% | PEG 22% | Control | PEG 16% | PEG 22% | Control | PEG 16% | PEG 22% |
| Taezhnaya | 83,8 | 56,4* | 50,0* | 43,3 | 43,6 | 30,6 | 32,4 | 33,3 | 16,7*[a] |
| Novosibirs-kaja 15 | 76,0 | 37,5* | 25,0* | 44,0 | 75,0* | 39,1[a] | 36,0 | 58,3* | 16,7*[a] |
| KS-1607 | 84,6 | 50,0* | 75,0[a] | 46,2 | 50,0 | 25,0*[a] | 38,5 | 33,3 | 8,3*[a] |
| Minusa | 77,3 | 60,9 | 66,7 | 65,9 | 47,8* | 42,9* | 59,1 | 34,8* | 16,7*[a] |
| Average data | 80,4 | 51,2* | 54,2* | 49,8 | 54,1 | 34,4*[a] | 41,5 | 39,9 | 14,6*[a] |

*- significant difference between control and selective media (P≤0,05);

a – significant difference between selective medium and medium with minimum selective level (P≤0,05).

**Table 5. Growth parameters of Minusa, KS-1607 and their regenerants plantlets under salt stress conditions (NaCl 1,68%)**

| Genotype | Root length Σ, mm | | Shoot length, mm | | Root weight, mg | | Shoot weight, mg | |
|---|---|---|---|---|---|---|---|---|
| | control | NaCl | control | NaCl | control | NaCl | control | NaCl |
| Minusa | 423,1 | 61,9 | 151 | 21,8 | 78,6 | 28,7 | 85,6 | 31,3 |
| RS(Minusa)3 | 317*** | 89,3** | 121** | 35,4*** | 57,9*** | 30,2 | 72,8* | 34,9 |
| RS(Minusa)9 | 469 | 77,6 | 158 | 40,8*** | 89,5 | 24,4 | 89,8 | 41,6** |
| KS-1607 | 296 | 35,5 | 129 | 7,59 | 83,0 | 15,9 | 105 | 15,2 |
| RS(KS-1607)1 | 292 | 62,7** | 154 | 31,8*** | 84,0 | 24,5** | 98,9 | 34,0*** |
| RS(KS-1607)2 | 387** | 46,1 | 137 | 14,7 | 95,0 | 18,2 | 95,6 | 24,8* |
| RS(KS-1607)3 | 345 | 38,6 | 128 | 10,1 | 91,4 | 18,8 | 99,7 | 14,0 |

Significant difference between regenerant and parental variety * - P≤0,1; ** - P≤0,05; *** - P≤0,01.

Immature embryos were used in research. So the question of their age, which can ensure the highest regenerant yield, appeared. The embryo age didn`t influence on callus induction frequency.

But the quantity of samples with organogenesis was 2-2,5 time higher in culture of more mature embryos than in three week age ones under these conditions. As the result the embryos taken at 20-21 days after the beginning of the earing have showed higher morphogenesis and regenerant yield in comparison with the material that had been taken at milky-wax ripeness stage (27-28 days) (Table 2). It seems that less differentiated embryos are more sensitive to exogenous hormones level change in comparison with more mature material. As the result the less mature embryos almost don`t formed any growing-points during callusogenesis step. Their calluses easier formed organogenic areas and regenerants after 2,4-D level decrease on proliferation medium in addition.

Different regeneration media compositions were tried to increase regenerant yield. It was detected that the media with IAA (1 mg/l) and kinetin (2 mg/l) (no. 13) [[7]] or BAP (2 mg/l) (b) [[11]] are the best for organogenesis stimulation under nonselective conditions. The highest regeneration was detected under stress influence at the medium with higher level and ratio of IAA (1,75 mg/l) and kinetin (10 mg/l) (no. 14) [[9]] (Figure 1).

## OPTIMIZATION OF LEVELS OF SELECTIVE AGENTS

Influence of different levels of NaCl (Table 3) and PEG (Table 4) on wheat callus proliferation and plant regeneration was investigated to increase effectiveness of stress-tolerant varieties selection.

NaCl concentration of 0,42% didn`t ensure significant suppression of growth. This aim was reached by increasing the NaCl level up to 0,84%. The content of salt 1,68% almost stopped organogenesis and resulted in zero regenerant yield in callus culture of all genotypes except KS-1607 (Table 3)

PEG is used widely in callus culture drought-tolerant varieties selection [[12], [9], [10], [2]].

**Table 6. $K_{red}$ of acid-tolerant regenerants (RA), regenerants created on control medium (RC) and parental genotypes**

| Parametrs | | Minusa | RA (Minusa) | Taezhnaja | RC (Taezgnaja) 1 | RC (Taezhnaja) 2 |
|---|---|---|---|---|---|---|
| $K_i$ | $K_l$ | 0,47 | 0,60 | 0,18 | 0,29 | 0,31 |
| | $K_{wp}$ | 0,52 | 0,71 | 0,20 | 0,36 | 0,36 |
| | $K_{wr}$ | 0,32 | 0,51 | 0,09 | 0,19 | 0,23 |
| | $K_{ws}$ | 0,68 | 0,80 | 0,26 | 0,45 | 0,41 |
| $K_{red}$ | | 0,51 | 0,67 | 0,18 | 0,32 | 0,33 |

Two PEG concentration influence on wheat callus culture growth and development were investigated (Table 4). The level of PEG of 22% induced stable decreasing of regeneration capacity. However, leafs formed under that conditions looked glassy and were easy affected by fungal diseases. The lower PEG concentration didn`t induced any changing of culture growth parameters in comparison with control conditions. The level of PEG 18% was considered suitable for selection *in vitro* as a result of our research and according to research data of other scientists [[13]].

## MULTIPLE REGENERATION PHENOMENON

Regenerant yield is one of the main characteristic of callus culture methods applying effectiveness. Regenerants yield can be also increased due to multiple regeneration phenomenon (MRP) (Figure 2). The higher frequency of MRP at late cultivars callus culture in comparison with early ones was showed for barley [[14]]. There is no similar data for wheat. Frequency of MRP in early wheat cultivar`s (Taezhnaja, Novosibirskaja 15) callus culture was twice as higher as in late cultivar`s (Minusa, KS-1607) one, 4,1±0,81 and 1,56±0,27%, respectively. It seems that separate regeneration sites can more often organized in several centre of attraction because of early genotypes original high development rate.

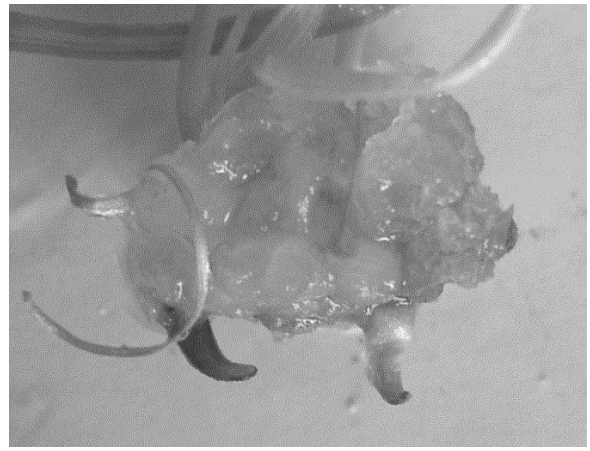

Figure 2. Callus with MRP.

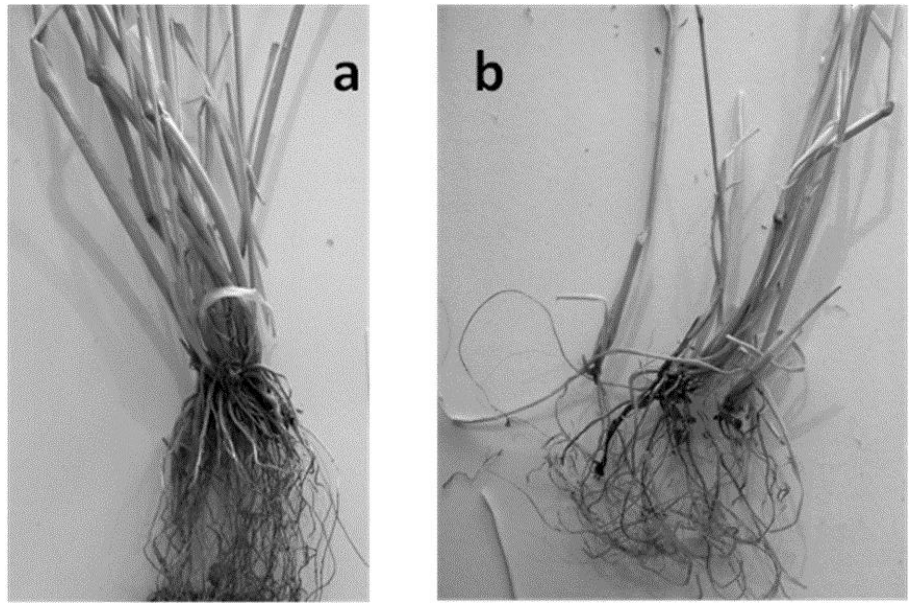


Figure 3. Roots of the wheat regenerants without RSMRP (a), with RSMRP (b).

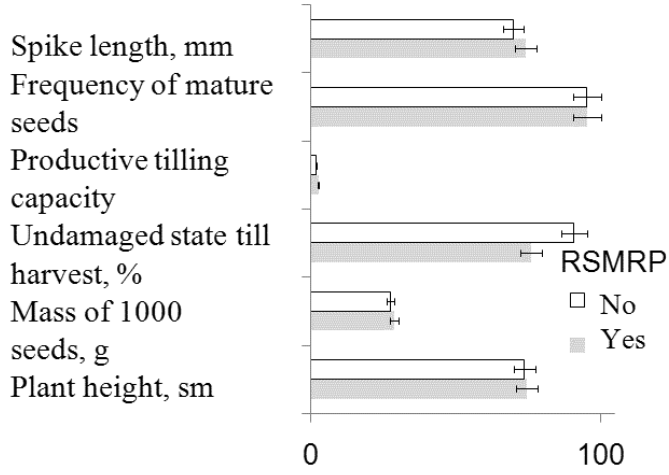


Figure 4. Influence of RSMRP presence at F0 of regenerant lines on characteristic of F1 plants, grown in the field.

The regenerants of early genotypes had several tillering node in their rootage – remained sign of MRP (RSMRP) - under greenhouse conditions after transplanting of single regenerant from test-tube to soil (Figure 3).

Regenerant lines with RSMRP at F0 showed the increase of productive tilling capacity at F1 under field conditions (Figure 4). Also there are some evidence of correlation between productive tilling capacity of explants donor plant and MRP frequency of callus culture [[15]]. So it seems that frequency of MRP is an original feature of genotype expressed in plant productive tilling capacity in the field.

## PHYSIOLOGICAL TOLERANCE TESTS OF REGENERANT LINES CREATED ON SELECTIVE MEDIA

Seeds of 26 regenerant lines created on selective media and of 20 lines created on nonselective media were obtained. The third generation of regenerants was tested for salinity (NaCl 1,68 %) or acidity (pH 3,0) tolerance according to the selection direction.

## COMPARATIVE CHARACTERIZATION OF SALINITY TOLERANCE

Regenerants of KS-1607 and Minusa varieties created on medium containing salt (NaCl 0,42%) were more tolerant under stress conditions according to the test data (Table 5).

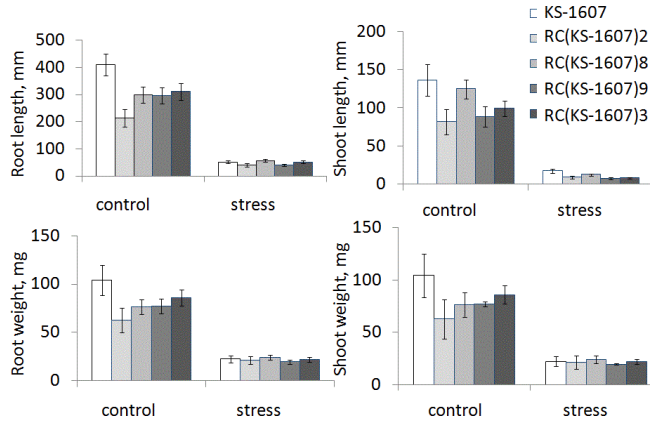


Figure 5. Influence of NaCl (1,68%) on plantlets growth of KS-1607 and it`s regenerants, created on control medium (RC).

The regenerants created on control medium also showed some tolerance – decrease of their growth activity in salt solution in comparison with data in clear water was lower than parental genotype KS-1607 one`s (Figure 5). This feature of RC regenerants results from presence of selection for cells with high regeneration activity under callus culture conditions.

Difference in reaction of samples (regenerant lines) can be explained by intragenotype polymorphism of parental cultivar as well as by somaclonal variations that are the base of all *in vitro* selection of stress-tolerant varieties technologies [[16], [1], [17]]. It seems that we can talk about inherited features created under callus culture conditions because they are showed in the tests conducted on third generation.

## ACIDITY TOLERANCE CHARACTERIZATION

Growth stimulation of plantlets was detected when roll test of acid tolerance was used.

Filter paper and wheat seeds increase medium pH up to not stress level during germination.

We suggest: 1) to conduct acidity tolerance tests on nonreactive substrate and replace the medium every day; 2) introduce into method coefficient of tested plants organ growth function reduction in stress conditions in comparison with control one.

$$K_{red} = \frac{K_{wr} + K_{ws} + K_{wp} + K_l}{4},$$

where average root weight ($K_{wr}$), shoot weight ($K_{ws}$), plant weight ($K_{wp}$) and shoot length ($K_l$) are the particular coefficients of growth reduction result from ratio of average value under stress conditions to the average value under control ones. The higher coefficient is so more tolerant the variety is. The parameter of root length was excluded from the method because of tendency of acidity tolerant varieties to shorten these organs under stress conditions parallel with increasing of root system branching.

The improved method was used in physiological test of acidity tolerant regenerant created in Minusa callus culture on selective medium with pH 4,0. That test showed the technology of *in vitro* selection effectiveness even more clearly than in the tests of salinity tolerance and confirmed the inheritance of acidity tolerance feature.

The growth activity decrease of regenerant was lower than Minusa plantlets showed under stress conditions in comparison with control ones, which resulted in coefficients $K_{red}$ 0,671 and 0,504 respectively (Table 6).

The improved test method allowed retracing the dynamic of growth which showed the superiority of regenerant over its parental cultivar (Figure 6).

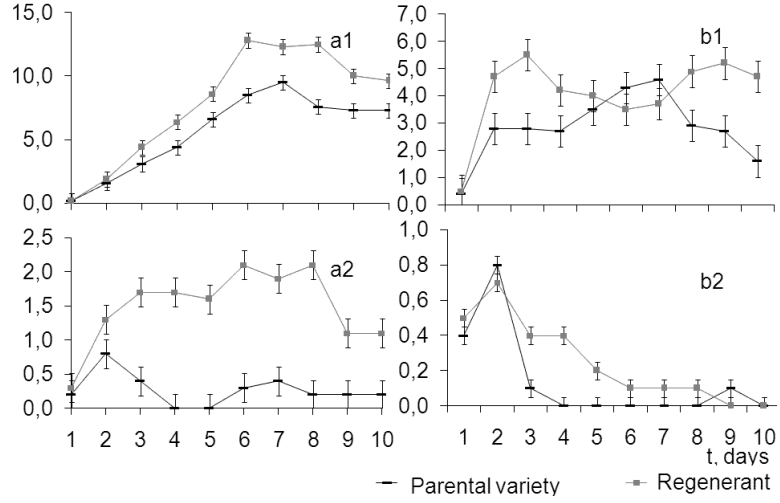


Figure 6. Rate of Minusa and its regenerant plantlets shoot (a) and root (b) elongation under control (1) and stress (2) conditions (V, mm/day) (P≤0,05).

Germination rate of regenerant`s root elongation was 1,6 times higher than parental cultivar one`s under control conditions in second day. This ratio was reached by the eighth day in variant with pH 3,0. The longer active growth stage of regenerant plantlets than parental one`s - till the eighth day, under stress conditions was detected. Root growth of parental cultivar stopped by the fourth day under pH 3,0.

All these data confirm the tolerance of regenerant to high $H^+$ concentration. Also it is important that regenerant surpass the parental variety under control conditions. The major part of ploughed field has neutral soil conditions. So the regenerant superiority in these conditions can be the determinant factor in selection material choosing.

Regenerants of Taezhnaja created on control medium also showed high level of tolerance in comparison with their parental variety (Table 6).

## CONCLUSION

Spring soft wheat varieties *in vitro* selection for drought, salinity and acidity tolerance technology was developed at the base of the local Siberian breeding material. Medium hormone composition and selective agent levels were optimized. Explants characteristics were established and most sensitive to callus culture conditions genotypes were chosen.

Some mechanisms of spring soft wheat callus culture development were detected. Multiple regeneration phenomenon of wheat was investigated for the first time. The correlation of this phenomenon with donor plant and regenerant tilling capacity and their vegetation period was detected.

Method of cereal crops test for acidity tolerance was improved. High tolerance to soil salinity and acidity of regenerants created on selective media was showed in physiological tests. So the effectiveness of developed *in vitro* selection technology was confirmed.

Three regenerants were transfered to prior varieties test department according to the research data of two-year field experiments.

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*Chapter 9*

# STRUCTURAL INVESTIGATION OF THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND ACID GELATIN IN WATER

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## ABSTRACT

This work studies interactions between a small globular protein and a relatively large protein with a conformationally variable chain using dynamic light scattering, differential scanning calorimetry, circular dichroism, fluorescence, and absorption measurements. It uses the dilute and semidilute system water/bovine serum albumin (BSA)/acid gelatin as a model. Gelatin molecules are able to form interpolymeric complexes with BSA in water at the temperatures above the temperature of the conformation transition and BSA/gelatin ratio ~6:1 (mole/mole). Interpolymer interaction leads to collapse gelatin macromolecules due to their lost of the total negative charge, partial stabilization of the secondary structure (increase the mean helix content), and stabilization of BSA molecules against thermo aggregation. At the same time, the thermo aggregation process of BSA molecules passes ahead of their thermodenaturation process.

**Keyword:** Bovine serum albumin, acid gelatin, interaction, structure formation, protein aggregation, conformation changes

## INTRODUCTION

Investigations on the mechanism of complexation and the molecular characteristic of protein-protein complexes play an important role in biotechnology and various fields of pure and applied science[1]. When two or more proteins_bind together, often to carry out their

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biological function. Many of the most important molecular processes in the cell such as DNA replication are carried out by large molecular machines that are built from a large number of protein components organised by their protein-protein interactions [2,3]. Protein interactions have been studied from the perspectives of biochemistry [4],quantum chemistry [5], molecular dynamics [6], signal transducton [7], and other metabolic or genetic/epigenetic networks. Indeed, protein–protein interactions are at the core of the entire interactomic[8] system of any living cell. The interactions between proteins are important for the majority of biological functions. For example, signals from the exterior of a cell are mediated to the inside of that cell by protein–protein interactions of the signaling molecules [9]. This process, called signal transduction plays a fundamental role in many biological processes and in many diseases (e.g. cancers). Proteins might interact for a long time to form part of a protein complex, a protein may be carrying another protein (for example, from cytoplasm to nucleus or vice versa in the case of the nuclear pore [10] (importins), or a protein may interact briefly with another protein just to modify it (for example, a protein kinase will add a phosphate to a target protein [11]). This modification of proteins can itself change protein–protein interactions. For example, some proteins with $SH_2$ domains only bind to other proteins when they are phosphorylated on the aminoacid tyrosine while bromodomains specifically recognise acetylated lysines [12]. Protein–protein interactions are of central importance for virtually every process in a living cell. Information about these interactions improves our understanding of diseases and can provide the basis for new therapeutic approaches. Protein mixtures may exist in molecular dispersed and colloidal dispersed states depending on the properties of the individual proteins, the composition of the mixtures and the conditions in which the mixtures are obtained. Colloidal protein dispersions play a pivotal role in our everyday life and can be exploited for the efficient production of food [13]. The interaction between proteins has been extensively investigated, in particular for the modulation of living processes in plant and animal cells, These interactions may result in the formation of water-soluble complexes, complex coacervation, formation of amorphous precipitates, stabilised by diferent type of bonds , or in the thermodynamic incompatibility of the macromolecules leading to liquid-liquid phase separation. Protein-protein interaction occur in many physical and chemical processes and affect nutritional and organoleptic quality of food products during manufacture and storage. Most studies, to date, display interesting and technologically useful properties produced by interactions including: enchanced gelation properties by synergistic interactions, phase separation and new textural properties and aggregation of oppositevily charged proteins [14]. Clearly, a knowledge of protein interaction can lead to a better understanding of biochemical changes in food products during processing and storage such as aggregation of proteins in fish leading to toughening on frozen storage [14]. Furthemore, an understanding of the effect of protein structure on protein-protein interactions, for example, of smooth and skeletal muscle proteins, permit the manipulation of protein side chains in order to enhance gelation properties. However, data are lacking for a quantitative understanding of how structural features of the proteins, as well as the diferent physico-chemical factors affect molecular, and aggregation properties of complexes. The aim of this study to characterize intermcromolecular interactions, structure and aggregation in the system containing a small globular protein and a relatively large protein with a conformationally variable chain using dynamic light scattering, differential scanning calorimetry, circular dichroism, fluorescence, and absorption measurements. It uses the dilute and semidilute system water/bovine serum albumin (BSA)/acid gelatin as a model. The molecular weight, charge, structure and

topography of the accessible surface of water soluble complexes of proteins are differ markedly from the "free" proteins. Therefore it can be assumed that all these factors may affect the interaction. Most experiments were performed in the absence of low molecular salt to enable a comparison of the present data with rheological experiments with the single phase and two phase systems. Because thermal denaturation is usually accompanied by an aggregation of protein molecules leading to optical changes, the intensity of the transmitted light was also measured to study the thermal stability of BSA in solutions in the presence of gelatin. All measurements were performed after previous holding of biopolymer solutions and their mixtures during 12-15 h. Previous experiments shown that longer store of solutions before measurement not accompanied by the additional contribution to the data of measurements. BSA or plasma albumin is a well-known globular protein ($M_W$ =67 kDa) that has the tendency to aggregate in macromolecular assemblies. Its three-dimensional structure is composed of three domains, each one formed by six helices. 17 disulphide bonds are located in BSA molecule. The most common molecular form is a prolate ellipsoid (4.1 nm x 14.1 nm) [16]. Gelatin is a protein derived by thermal denaturation of collagen. The protein is unique in that it is made up of triplets of amino acids, gly-X-Y. The X and Y can be any amino acid but the most common are proline and 4-hydroxyproline, which have a five membered ring structure. This is most important because it lies at the root of gelatine's unique ability to form smooth, elastic, thermo-reversible gels [17]. These biopolymers are well known, widely used in industry for their textural and structuring properties [16,17], and their capacity to form complexes with polyelectrolytes is known from literature (see for example [18-21] ).

## 2. EXPERIMENTAL SECTION

### 2.1. Materials

The BSA Fraction V, pH 5 (Lot A018080301), was obtained from Across Organics Chemical Co. (protein content = 98-99%; trace analysis, Na < 5000 ppm, CI < 3000 ppm, no fat acids were detected). The isoelectric point of the protein is about 4.8-5.0, and the radius of gyration at pH 5.3 is equal to 30.6 Å.[22] The water used for solution preparation was distilled three times. Most measurements were performed at pH 5.3. The extinction of 1% BSA solution at 279 nm was *A*1cm 279) 6.70, and that value is very close to the tabulated value [23] of 6.67. For the molecular weight of BSA, we use 69 kDa (582 amino acid residues), in accordance with amino acid analysis data.[24]

The gelatin sample used is an ossein gelatin type A 200 Bloom PS 8/30 (Lot 09030) produced by SBW Biosystems, France. The Bloom number, weight average molecular mass and the isoelectric point of the sample, as reported by the manufacturer, are, respectively, 207, 99.3 kDa and 8–9. Since the commercial sample contained traces of peptides and various substances regarded as impurities, an additional purification by washing with deionised water for 3 h at 5$^{o}$C, was used. The major characteristics of the purified gelatin were described in a previous paper [25]. To prepare molecularly dispersed gelatin solutions, deionized water was gradually added to the gelatin, and stirred first at 60$^{o}$C for 20 min and then at 40$^{o}$C for 1 h. The required pH values of the solutions (5.4) was adjusted by addition of 0.1–0.5 M NaOH or

HCl. The resulting solutions were centrifuged at 50,000 g for 1 h at 40°C to remove insoluble particles. The ternary water–gelatin–BSA systems with required composition were prepared by mixing solutions of each biopolymer at 40°C. Both the BSA and the dextran solutions show Newtonian behavior (at temperatures of 40°C and at shear rates up to 30 $s^{-1}$) in the concentration range from 25 to 30 wt % within which the hydrodynamic volumes of the different macromolecules already overlap.

## 2.2. Dynamic Light Scattering

Determination of Intensity- size distribution, volume-size distribution of hydrodynamic radii, ($R_H$) and zeta potentials of BSA, gelatin, and their mixtures was performed, using the Malvern Zetasizer Nano instrument (England), using a rectangular quartz capillary cell. Concentration of one protein in BSA/gelatin mixtures frequently was kept at 0.25 (w/w). For each sample the measurement was repeated 3 times. The samples were filtered before measurement through DISMIC-25cs (cellulose acetate) filters (sizes hole of 0.22 μm for the binary water-protein and solutions and 0.80 μm for the BSA-gelatin mixtures. Subsequently the samples were centrifuged for 30 seconds at 4000 g to remove air bubbles, and placed in the cuvette housing. The detected scattering light intensity was processed by Malvern Zetasizer Nano software.

## 2.3. Circular Dichroism Measurements

CD measurements of BSA solution alone and in the presence of gelatin were performed using a Chiroscan Applied Photophysics instrument. Far-UV CD spectra were measured in 1-mm cells at 20°C and 40°C. The solutions were scanned at 50 nm min-1 using 2 s as the time constant with a sensitivity of 20 mdeg and a step resolution of 0.1. The ellipticity at 222 nm, $\tilde{o}222$, is assumed to be linearly related to mean helix content, $f_H$, which can be calculated from the Lifson-Roig-based helix-coil model according to the procedure described before by Luo and Baldwin [26,27].

## 2.4. Fluorescence Measurements

Fluorescence emission spectra between 280 and 420 nm were recorded on a RF 5301 PC Spectrofluorimeter (Shimadzu, Japan) at 25 °C and 40°C with the excitation wavelengths set to 250, 270, and 290 nm, slit widths of 3 nm for both excitation and emission, and an integration time of 0.5 s. The experimental errors were approximately 2%.

## 2.5. Turbidimetry

The cloud points of aqueous BSA solutions and their mixtures with gelatin solutions were determined by measuring the transmittance of the solutions as a function of temperature for

different BSA/gelatin weight ratio (q) using CARY 300 Bio UV-visible spectrometer. The samples were heated up to temperatures 40$^{o}$C, 50$^{o}$C,52$^{o}$C, 54$^{o}$C, 56$^{o}$C and 58$^{o}$C before measurements.

### 2.6. High-Sensitivity DSC

Thermal denaturation of BSA in aqueous solution in the absence and in the presence of dextran was monitored with a highly sensitive differential scanning calorimeter, model DASM-4, Puschino, Russia.

Thermograms were obtained between 20 and 90$^{o}$C, at a scan rate of 60 $^{o}$C h$^{-1}$. Previous experiments performed at a scan rate of 20$^{o}$C h-1 showed the same curves, indicating insignificant contributions from kinetic effects. For all the measurements, the pH values was 5.4. Some test experiments performed at different concentrations of BSA (0.075 mol dm$^{-3}$ – 0.149 mol dm$^{-3}$) produced essentially the same curves, ruling out significant contributions from concentration effects. All results are averages of three independent measurements.

Degassing during the calorimetric experiments was prevented by additional constant pressure of 1.7 atm over the liquids in the cells. At first, the water was placed in the sample and in the reference compartments. A DSC curve corresponding to a water vs water run was used as the instrumental baseline for BSA solution.

## 3. RESULTS

### 3.1. Hydrodynamic Radiuses and Zeta Potentials of BSA, Gelatin and their Mixtures

We focus our attention to the interaction between BSA and gelatin in aqueous solutions at pH 5.4 within the region of pair interaction. The scattering intensity as a function of size for 0.25 wt% solutions of gelatin and BSA is shown in Figure 1. Z-Average diameter of BSA at given conditions is about 9 nm that is close to that (8.39 nm) reported on the web site of the California NanoSystems Institute (CNSI). About 90 % of all particles of BSA has a average diameter 7.4 nm (Figure 1). The sample of gelatin is strongly polydisperse but the main peak ( 50 %) has the average diameter 280 nm. Gelatin sample also contain small parts of the low molecular weight fractions with the average diameter 7.6 nm (27 %) and 18 nm (23%).

The scattering intensity as a function of BSA/gelatin weight ratio (q) at pH 5.4 is presented in Figure 2. The concentration of gelatin in mixture was kept 0.25 wt%.

Since the main peak of the gelatin sample at 140 nm place far enough from the main BSA peak we shall consider mainly possible changes of the main gelatin peak in the presence of BSA. As can be seen from Figure 1 the presence of small amount of BSA in the gelatin solution (at q =0.2 and 0.4) do not affect on the intensity of the main gelatin peak. However at a higher q the intensity of this peak first decrease considerably (q=1.0) and then completely disappear at q=2.0. At such compositions the new big peak appear corresponding complex particles with the Z average diameter 28 nm. At a higher content of BSA in the mixture (at q=8) the shoulder is appear which correspond to “free” BSA molecules. In order understand

the reason of collapse gelatin molecules during their interaction with BSA we have to consider possible changes of the zeta poteintial for gelatin molecules in the presence of BSA. The corresponding data are presented in Figure 3. As can be seen from Figure 3 increase of BSA content in the BSA/gelatin mixture leads to decrease of the total positive charge of gelatin molecules up to zero at q=6.The fall of the zeta potential is especially considerable starting from q=2. At this q values the most part of gelatin is involved in interaction with BSA (Figure 2).

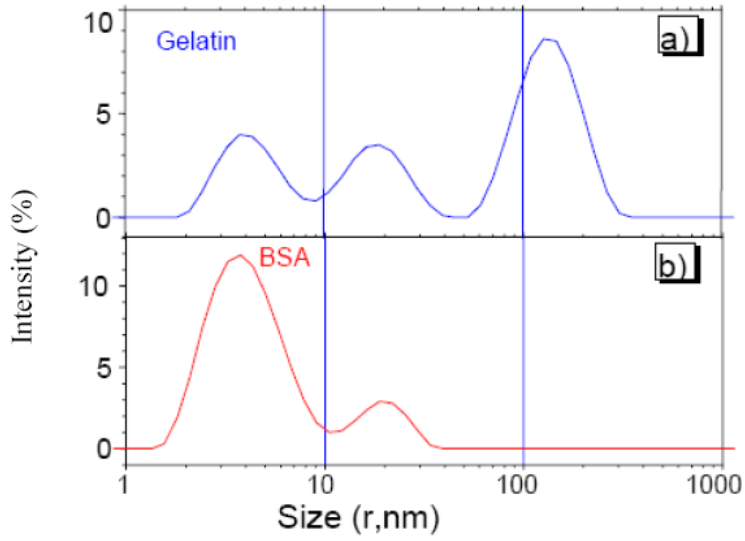


Figure 1. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

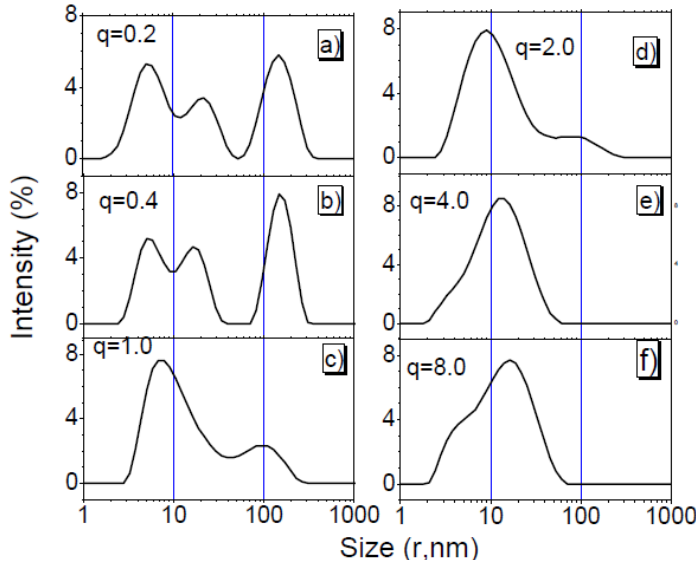


Figure 2. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

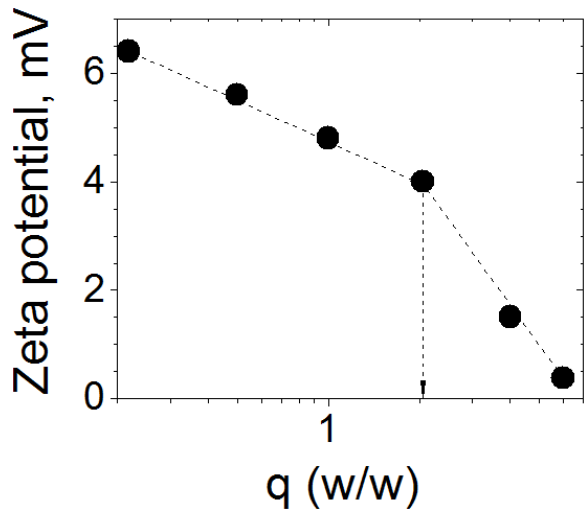


Figure 3. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

## 3.2. Effect of Gelatin on the Secondary Structure of BSA (CD Spectra)

CD can provide information about the secondary structure of proteins and nucleic acids and about the binding of ligands to these types of macromolecules [28,29]. Figure 4 shows far-UV CD spectra at 40°C for BSA and gelatin in water. Gelatin at +5°C had a positive maximum peak at 221nm and a negative minimum peak at 192 nm, suggesting a typical triple helical conformation, [30] whereas the positive peaks in gelatin at 40°C disappeared, showing the random coils configuration.

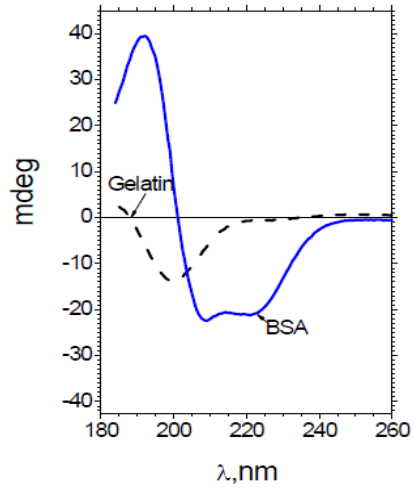


Figure 4. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

However, the triple helix can partly reform for gelatin when the temperature of the sample falls below the gelation temperature.[31] The triple helix of native collagen can transform to the random coil configuration when it is heated above the denaturation temperature. In the denaturation process, the physical properties such as viscosity, solubility and optical activity changed due to the collapse of the triple helical structure. CD spectra and viscosity change are widely used to determine the denaturation temperature of native collagen. The helix-coil transition of collagen involves the breakage of hydrogen bonds between the adjacent polypeptide chains of collagen molecules in the denaturation process. The intact trimers ( ) of collagen turn into individual chains (α) or dimers (β) in the transition, causing the decrease of viscosity at the same time.

At 40 $^{o}$C BSA contains 51 % of alpha helical structure. The mean helix content, $f_H$ (in % ) for BSA calculated according to [26,27].

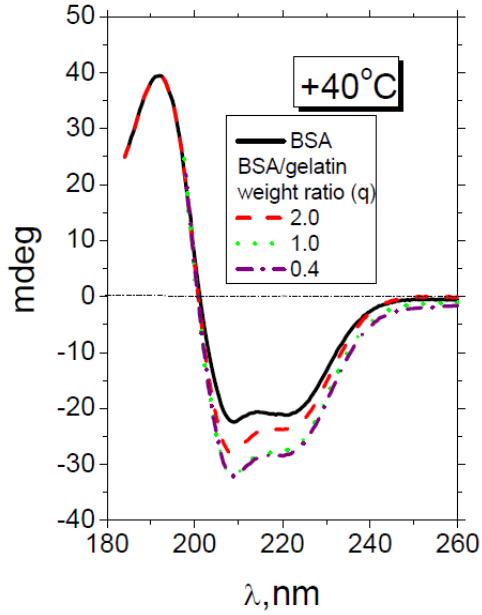


Figure 5. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

The addition of gelatin to BSA solution at BSA/gelatin weight ratios ranging from 2.0 to 0.4 leads to significant increase in the negative band at 222 nm and significant increase in the negative maximum at 209 nm as shown in Figure 5. Such a increase in the $\theta_{222}$ values corresponds to an increase in the content of alpha-helical structures.

## 3.3. Conformational Changes (Fluorescence Measurements)

Figure 6 shows a typical fluorescence spectrum for an aqueous BSA solution at the excitation wavelengths ($\lambda_{exc}$) = 250 nm, 270 nm and 290 nm and 40$^{o}$C. The wavelength of maximum emission ($\lambda_{max}$) was about 339 nm, regardless of $\lambda_{exc}$ in the range 250-290 nm. The presence of even small amount of gelatin in BSA solution leads to a visable leads to a

visible decrease in the fluorescence intensity. The decrease in the intensity is especial remarkable at 270 nm at a highest content of gelatin in BSA solution (at q=0.4). At the same time we did not observed any appreciable shift for a position of the maximum of the fluorescence intensity at any q values.

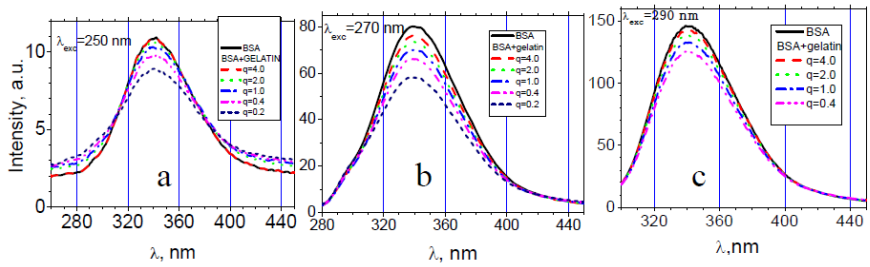


Figure 6. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

## AGGREGATION OF BSA

Since the aggregation tendency of BSA upon heating at pH 5.4 (i.e. slightly above the pH of IEP /4.8-5.0/) depends strongly on its concentration, the effect of the presence of gelatin in BSA solution was monitored at constant concentration of BSA by the absorbtion of light at 500 nm and DLS measurements. Figure 7. shows the experimental findings in terms of light adsorbtion.

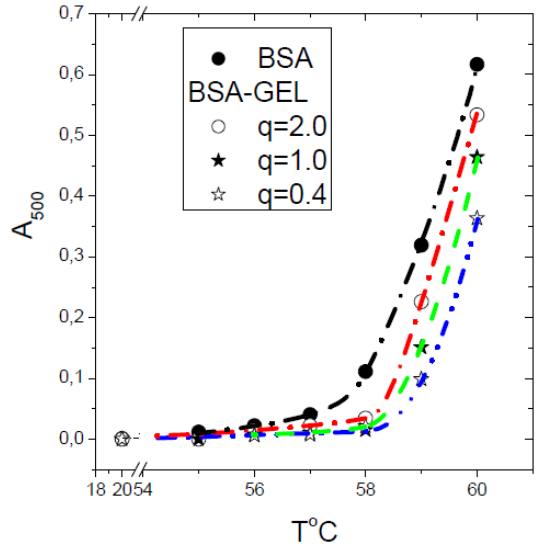


Figure 7. Antonov et al. “Structural investigation of the interaction between bovine serum albumin and acid gelatin in water”.

The findings can be interpreted in the following manner: The absorbtion of light of BSA abd BSA/gelatin solutions decreases as a function of temperature, because of progressing aggregation. In the binary solutions BSA aggregates as T becomes higher than 56°C. The

thermoaggregation process is observed in a narrow temperature range, and the absorbtion values becomes very high already at 60°C. The degree of reversibility of thermoaggregation depends on the final temperature of heating and the time required for the temperature cycles [20]. An increase of the residence time at the higher temperatures increases the degree of irreversibility.

For sufficiently rapid heating-cooling processes (1°C per minute and higher) and final temperatures, which are not too high, the extent of aggregation remains insignificant and the absorbtion reaches its initial value after cooling; the process can thus be considered to be reversible. For BSA concentration ≤ 1% wt the upper temperature limit amounts to 55°C. The present of even small amount of gelatin in BSA solution (For q = 4 (q=BSA/gelatin w/w) the critical aggregation temperatures increase significantly as shown in Figure 7. It can be seen from this graph that the critical aggregation temperatures increase monotonically from 55°C to 58°C when the concentration of gelatin in mixture increases (when q decrease from 4 to 0.4).

In order to quantify the effect of gelatin on aggregation of BSA, we have performed DLS experiments for solutions of BSA, and the their mixture with gelatin at q =1.

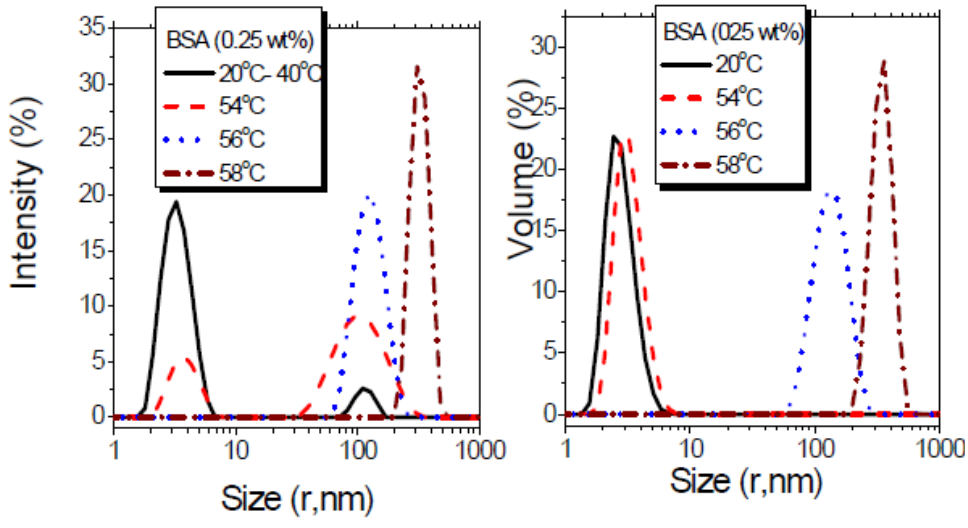


Figure 8. Antonov et al. "Structural investigation of the interaction between bovine serum albumin and acid gelatin in water".

At these conditions the most of BSA molecules interacts with gelatin molecules (Figure 2) The scattering intensity as a function of size and the volume fraction as a function of size for 0.25 wt% BSA is shown in Figure 8. One can see the thermoaggregation of BSA takes places starting from 54°C and at 56°C almost all BSA molecules form a large (> 200 nm in size) thermoaggregates. The thermal behavior of the BSA/gelatin mixture is differ from that of BSA solution (Figure 9). In this case thermoaggregation process start the only at 58°C, but even at this temperature the only small part of BSA molecules form a large aggregates (Fig 9 b). The results demonstrate that the presence of gelatin increase considerably stability of BSA molecules against thermoaggregation.

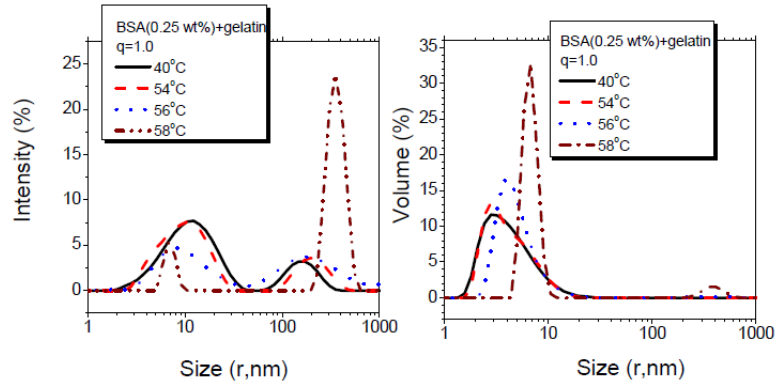


Figure 9. Antonov et al. "Structural investigation of the interaction between bovine serum albumin and acid gelatin in water".

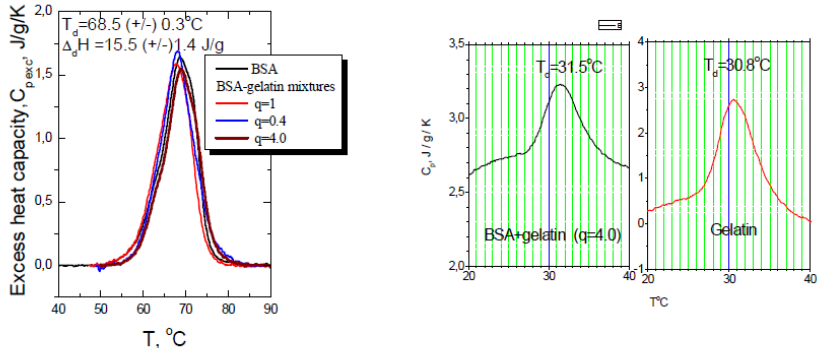


Figure 10. Antonov et al. "Structural investigation of the interaction between bovine serum albumin and acid gelatin in water".

## 3.4. Thermal Denaturation (DSC Measurements)

The DSC trace of thermal denaturation of BSA alone and in the presence of gelatin at different q values at pH 5.4 is shown in Figure 10. The reversibility of the unfolding process was checked by reheating the sample after it had been cooled to room temperature in the calorimeter. If the BSA solution is heated up to 100 °C or higher denaturation is irreversible.

However, as judged by the area of DSC curve, the denaturation should be essentially reversible, provided the first heating was not carried on above the temperature at which the denaturation is effectively complete. Sturtevant and co-workers,[32-34] , Sanchez-Ruiz, [35] and Galisteo et al [36] have shown that the usual thermodynamic evaluation methods are applicable in cases where the irreversible steps do not take place during the time the protein spends in the temperature range of DSC transition, but occur at somewhat higher temperatures. It is thus permissible to apply equilibrium thermodynamics for the evaluation of thermodynamic parameters as function of the BSA/gelatin weight ratio. The thermal scan of for the BSA solution yields a symmetric peak (Figure 10). The calorimetric data were analyzed using the MicroCal Origin software, according to the methodology recommended by JUPAC [37] . The parameters obtained in this manner are collected in Table 1.

Thermodynamic functions of protein denaturation were determined by least-squares fits of the excess heat capacity data, using EXAM software as developed by Kirchoff [38].

The thermodynamic parameters of the one component transition for the thermal unfolding process of BSA as obtained from DSC analysis are presented in Table 1. Each value presents the average of three experiments and uncertainly represents the standard error of the mean. The $T_m$ values have an experimental error of 0.57 and calorimetric entalpy $\Delta H_{cal}$ values have a maximum expected error of $\pm$ 5% including errors in sample preparation, calibration constant and reproducibility. The van´t Hoff enthalpy ($\Delta H_{vH}$) and the cooperativity $\eta$, are also listed in Table 1. $T_m$ is the thermal midpoint; $T_{1/2}$ - the width of the peak at half-height; $\Delta H$- the area under the peak, which represents the enthalpy of transition for reversible processes; $\Delta H_{vH}$ - the van´t Hoff enthalpy; $\eta$ - cooperativity which is the ratio of calorimetric enthalpy to van´t Hoff enthalpy; $\Delta S_{cal}$ -denaturation entropy. The values of $T_m$ for such system are about 68.5 +/- 0.3$^o$C, and $\Delta H$ equals 1000-1100 kJ mol$^{-1}$, According to Michnil ( ) such behaviour is typical for BSA sample containing trace of fatty acids. It has been shown [39] that the single endotherm is well approximated as the sum of three independent two-state transitions.It is interesting to note that the endotherm peak for the BSA solution, and for the BSA(1.0%)-gelatin(1.0%) system appears at temperatures higher than 50$^o$C.

**Table 1. Thermodynamic parameters obtained from DSC scans of BSA alone and in the presence of gelatin at pH 5.4**

| System | $T_m$($^o$C) - ($^o$K) | $\Delta T_{(1/2)}$ | ($\Delta H_{cal}$), J g$^{-1}$ kJ mol$^{-1}$ | ($\Delta H_{vH}$), kJ mol$^{-1}$ | $\eta$ | ($\Delta S_{cal}$) J mol$^{-1}$K$^{-1}$ |
|---|---|---|---|---|---|---|
| BSA | 68.6 - 341.6 | 9.1 | 16.8 - 1122 | 379.8 | 2.95 | 3285 |
| +gelatin q= 0.4) | 68.1 - 341.1 | 9.1 | 15.5 - 1035 | 421.5 | 2.46 | 3035 |
| +gelatin (q=1.0) | 68.1 - 341.1 | 9.4 | 16.8 - 1122 | 366.2 | 3.06 | 3290 |
| +gelatin (q=4.0) | 68.8 - 341.8 | 9.2 | 15.9 - 1062 | 380 | 2.79 | 3107 |

# 4. DISCUSSION

## 4.1. Behaviour of BSA Molecules in Water

Although the behaviour of BSA molecules in aqueous solutions was subject of many studies, the results from literature differ due to different experimental conditions, composition of solvent, purity of the protein sample. The calorimetric and spectroscopic information is very sensitive to minor changes in the above conditions. Because the capacity of all proteins, in particular BSA, to interact with other polymers depends strongly on the local structural details of the components in water, we are in the following briefly dealing with the binary subsystem BSA/water**.**

The ratio of the intensities of the two minima of the molecular ellipticity at 222 nm and 209 nm (> 1) obtained for BSA solution at 25$^{o}$C (Figure 1) indicates that BSA belongs to the $\alpha/\beta$ class of proteins, i.e., consists of mixed segments of alpha-helical and $\beta$ -structure [40]. Literature data indicate that the secondary structure of BSA contains about 68-50% alpha-helices and 16-21% beta-sheets [41,42].

The comparison of the thermodynamic parameters presented in Table 2. for the thermal unfolding of BSA with those reported in the literature is difficult not only because of different experimental conditions but on account of different deconvolution procedures. The thermal denaturation of BSA in aqueous solution was characterised by a transition with $T_m$ =62$^{o}$C, $\Delta$H=335 kJ mol$^{-1}$ [43,44] thus by a similar temperature but lower enthalpy changes than reported here. The onset temperature of conformational changes, as found by DSC; is 58.1$^{o}$C [45] . Other available literature results, obtained under more dissimilar conditions gave: $T_m$=68$^{o}$C, $\Delta$H = 535-600 kJ mol$^{-1}$ (in 0.9% NaCI, pH 6.8 [46]; $T_m$ =61.5$^{o}$C, $\Delta$H = 568 kJ mol$^{-1}$ (in 0.067 M phosphate buffer, pH 6.0) [47,48], ; $T_m$=63.9$^{o}$C, $\Delta$H =785 kJ mol$^{-1}$ (in 0.1 M NaCI, pH 5.6) [49].

There are reports on the behavior of two different BSA samples in the literature. One is practically pure BSA globulin containing some traces of fatty acids [50] and with the second sample the fatty acids were also absent [39,51]. The former transition temperature was 69$^{o}$C-70$^{o}$C, that for the latter sample was about 62$^{o}$C. The melting of the native structure of the latter sample is the complex process which is described by two or three simple transitions overlapping in temperature. From our data (Figure 10) we can therefore conclude that we are working with the BSA sample containing some traces of fatty acids.

## 4.2. Behaviour of BSA Molecules in the Presence of Gelatin

The experimental findings demonstrate that the ratio of macromolecular components ( q) is the key factor determining the interaction of gelatin with BSA at pH 5.4 (natural pH values of pure BSA solutions in pure demineralized water) and the temperature above the conformation transition of gelatin.

Taking into account that the maximal binding of BSA and gelatin in complex takes place at q$\cong$ 2, knowing the weight-average molecular weights of BSA and gelatin we can roughly evaluate the BSA/gelatin molar ratio in the complex in the selected conditions. Simple calculation showed that at least 6 molecules of BSA join to 1 gelatin molecule, forming BSA/gelatin complex. The question is appears: what is the structure of the complexes formed? Usually, the sizes of interpolymeric complexes are larger then those of the interacting polymers [19]. However in the given case, the sizes of the complex particles are at least 10 times smaller then the size of gelatin molecules and approx. 50% larger then the sizes of the BSA molecules. It is reasonable to suggest that at given conditions complex formation results in the collapse of gelatin molecules due to sharp decrease of their total charge.The collapse of the gelatin macromolecules in solution at isoelectric conditions due to electrostatic attraction forces of oppositely charged groups has been shown early in study of the gelatin self association [52]. Figure 11. presents schematically particles of the BSA/gelatin complexes. The collapsed gelatin molecule wrap six BSA molecules forming the complex particle containing BSA molecules in more folded state then before interaction with gelatin.

At q values starting from 4 and below i.e in the presence of small amount of gelatin, BSA in mixture change the secondary and the local tertiary structures of BSA and especially its thermoaggregation behaviour. At the same time, the only small change in calorimetric enthalpy and co-operativity, in the presence of gelatin is observed (Table 1).

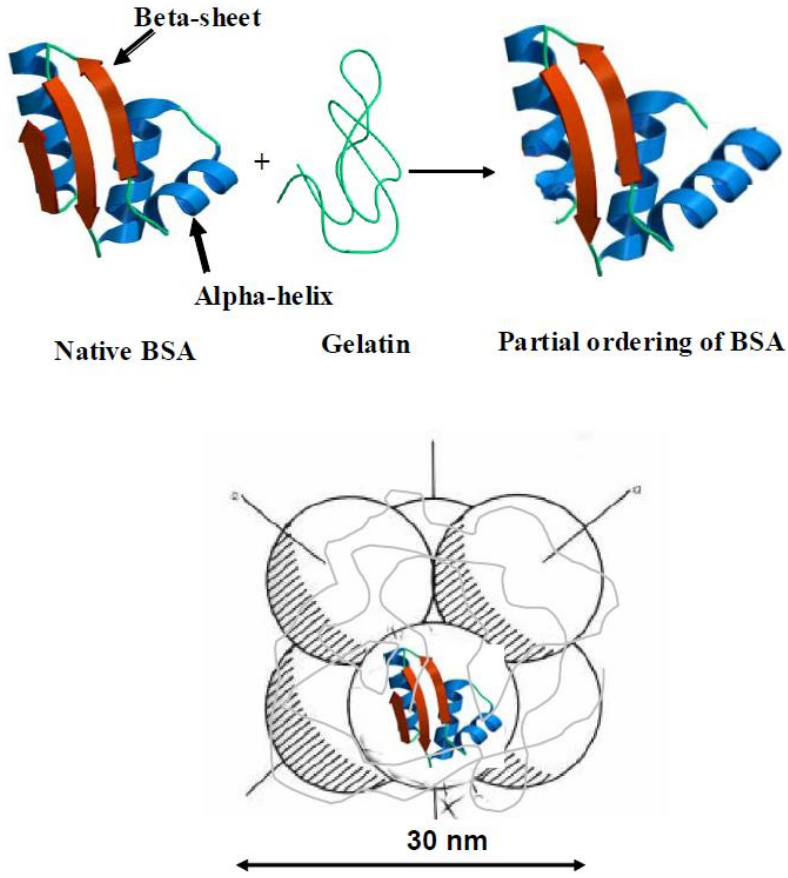


Figure 11. Antonov et al. "Structural investigation of the interaction between bovine serum albumin and acid gelatin in water".

An excess gelatin (1<q<0.4 ) induces slightly more significant structural changes. The partial folding, associated with an increase in alpha-helical content**,** shifts the onset temperature of thermoaggregation to higher temperatures. These changes are clear indication on an interaction between BSA and gelatin. This finding is consistent with turbidimetric and DLS results of the thermoaggregation study, which show that gelatin acts as a stabilizer at low /BSA/gelatin ratios. An excess of gelatin has an additional effect on the secondary structure of BSA. With decreasing q up to q=0.4 (cf. Figure 5) the ratio $\theta_{222}/\theta_{209}$ becomes less than unity; that observation clearly indicates the formation of $\alpha+\beta$ structures, i.e., BSA consists of separated segments of alpha and $\beta$ -structures.

The results of fluorescence analysis has shown quenching of fluorescence and the absence of any shift in the wavelength of the maximum of the intrinsic fluorescent emission that indicates on small changes in protein conformation. In proteins that contain all three aromatic aminoacids, fluorescence is usually dominated by the contribution of the tryptophan

residues, because both, their absorbency at the wavelength of excitation and their quantum yield of emission, are considerably greater than the respective values for tyrosine and phenylalanine. We consider the fluorescence quenching of BSA in the presence of gelatin in terms of local rearrangements of the tryptophan surroundings.

## CONCLUSION

Gelatin molecules are able to form interpolymeric complexes with BSA in water at the temperatures above the temperature of the conformation transition and BSA/gelatin ratio ~6:1 (mole/mole). Interpolymer interaction leads to collapse gelatin macromolecules due to their lost of the total negative charge, partial stabilization of the secondary structure (increase the mean helix content), and stabilization of BSA molecules against thermo aggregation.

At the same time, the thermo aggregation process of BSA molecules passes ahead of their thermodenaturation process, whereas these two phenomena accompany each other in the absence of dextran according to Privalov and Potekhin [53].

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*Chapter 10*

# THE EFFECT OF NANO- AND MICROPARTICLES ON PHASE SEPARATION AND THE STRUCTURE OF SEMIDILUTE BIOPOLYMER MIXTURES

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## ABSTRACT

We examine the issue of whether of low-volume fractions of nano- and microparticles can lead to phase separation in semidilute biopolymer mixture. To this end, we determine the phase diagrams and ESEM images of aqueous semidilute and weakly structured sodium caseinate-sodium alginate-dextran sulfate (SC-SA-DS) system in the presence of several the ultra clean charged micro particles with a diameter less, higher, and comparable with the size of the system network holes (2-3 um).We demonstrate that the last 3 um particles (both negatively and positively charged) help to enhance phase separation, and increase the viscoelastic properties of the emulsion, whereas the larger particles affect oppositely, and nanoparticles (210 nm and 910 nm in diameter) do not affect appreciably the phase separation and rheology. Experimental observations suggest that the dominant mechanism responsible for decrease thermodynamic compatibility in such system is perfect build 3 um particles into the holes (2-3 um in diameter) of the weak network of SC enriched phase and reinforcement of this network. Decrease in compatibility and increase in viscoelastisity are more pronounced by use highly charged hydrophobic carboxylate modified sulfonate latex (CLM-Sfn) and sulfate latex, and, in less degree, by use of aliphatic amino latex, and hydrophilic CLM latex.

**Keywords:** Biopolymer emulsion, demixing, particles, structure formation

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# 1. INTRODUCTION

The stabilization of emulsions by incorporating colloidal particles is known since one century with the pioneer works of Ramsden[1] and Pickering[2]. Stabilization and phase inversion in different oil-water emulsions by nano-silica particles has been recently intensively studied the research group of Binks[3-6]. Several mechanisms for stabilization of water–oil and oil–water emulsions by particles have been suggested, including steric (bilayer) stabilization of fully covered droplets[3], stabilization by particle monolayer's which can bridge droplets[4,7,8], effects of surface rheological properties[9,10] and even flocculation in the bulk[5].Vermant and co-workers[11], and Thareja and Velankar[12] has shown that particles at interfaces can be used to influence the structure and properties of polymer mixtures.The effects are similar to those in particle-stabilized water–oil emulsions. The size and shape of the dispersed phase of polymer blends depends on several processing parameters including rheology, interfacial properties, and the composition of the blend. This invariant morphology is due to a rapid establishment of equilibrium between drop breakup and coalescence. Recently, the effect of low-volume fractions of nanoparticles on the morphological processes of immiscible polymer blends was studied by Vermant and co-workers[13] and suppress coalescence for blends of poly-isobutylene and polydimethylsiloxane by silica particles was shown. More theoretically, Nesterov and Lipatov[14,15] studied the influence of fumed silica particles on the phase diagram behavior of polymer blends with lower critical solution temperature. They came to the conclusion that the total free energy of a blend system should also include the interaction parameters between the polymers and the inorganic filler surface. In other words, addition of a filler S to A–B blend stabilizes the morphology. Actually, the solid particles act as a compatibilizer by adsorbing A and/or B polymers on their surface. To play this role the inorganic phase should have the largest possible surface area and should be able to disperse very well in the two phases.

The reverse situation i.e. possibility of the phase separation in polymer mixtures induced by nano or micro particles is less clear because the experimental observations in this field are absent. This conclusion also concern multicomponent biopolymer systems. From a technological point of view, especially important are biopolymer systems which undergo liquid-liquid phase separation in a wide concentration range, starting from low concentrations[16,17]. Although the majority of biopolymer mixtures show phase separation, in most cases the phase separation takes places at critical total concentrations, which are much higher (7-12 wt%) compared with those of synthetic polymers (less than 1-2 wt%)[15].

In our resent paper[18] we described approach for inducing demixing of semidilute biopolymer mixtures by physical interactions of the constituents. The addition of sulfate polysaccharide to the semidilute protein-acid polysaccharide systems (dextran sulfate sodium salt/DSS/or carrageenans), even in trace concentrations ($10^{-3}$ wt %), lead to segregative liquid–liquid phase separation of the later**,** and a substantial increase in storage and loss moduli of the system.

The phase separation observed is the result of formation at pH 7.0 (i.e. far away from the iep of the caseins /4.4-4.6/) of DSS/SC water soluble charged associates (1:10 mol/mol), having $R_H$=0.26 um and electrostatic nature. The minimal compatibility of SC and SA was observed at the DSS/SC weight ratio of 0.14, which corresponds to an equality of the cationic

groups in the protein molecules and sulfate groups in DSS. At a higher SC concentration (4 wt %) SC-DSS associates forms a pore structure with a diameters of the holes of about 2-3 um. Such structure is usual for many water soluble protein-polysaccharide complexes. Data of chromatography indicate that DSS interacts first with SC associates having higher molecular weight. The degree of the protein conversion in the complex increases from approx. 30 % to 80% when the concentration of SC in the system grows from 1 to 2 wt %. Phase separation of semidilute ternary water-biopolymer 1-biopolymer 2 systems in the presence of DSS is observed here to be a rather common phenomenon, observed for different types of biopolymers, e.g. SC-gelatin type-A, gelatin type A-SA. Therefore the use of DSS as a decompatibilizer for semidilute biopolymer systems can find applications in processes for concentrating biological materials in two phase systems, because DSS induced demixing allows decreasing the critical concentration of the phase separation significantly.

It is important to note that complex formation of SC with DSS in dilute and semidilute solutions has been studied for the first time long ago[20] and recently in details by Dickinson [21,22]

The dominant mechanism controlling the phase separation involves the creation of water soluble protein-sulfate polysaccharide associates. Such systems forms the pore structure with a diameter of holes about 2-3 um. Thermodynamic incompatibility of biopolymers in such systems develop symbasis with the density of the network, and slop opposition with the diameters of the network holes[18].

In this work we examine the issue of whether of low-volume fractions of nano- and microparticles can lead to phase separation in semidilute biopolymer mixture. To do that, we determine the phase diagrams and ESEM images of aqueous semidilute and weakly structured sodium caseinate-sodium alginate-dextran sulfate (SC-SA-DSS) system in the presence of several micro particles containing the functional groups of different origin and sign of charge with a diameter less, higher, and comparable with the size of the system network holes (2-3 um).

We demonstrate that 3 um particles (both negatively and positively charged) help to enhance phase separation, and increase the viscoelastic properties of the emulsion. We found evidence that the dominant mechanism responsible for decrease thermodynamic compatibility in such system is perfect build 3 um particles into the holes (2-3 um in diameter) of the weak network of SC enriched phase, and reinforcement of this network.

## MATERIALS AND METHODS

The sodium caseinate sample (90% protein, 5.5% water content, 3.8% ash, 0.02% calcium) was purchased from Sigma Chemical Co. The isoelectric point is around pH = 4.7–5.2.The weight average molecular mass of the sodium caseinate in 0.15 M NaCl solutions is 320 kDa[18]. The medium viscosity sodium alginate, extracted from brown seaweed (*Macrocystis pirifera*), was purchased from Sigma. The weight average molecular weight of the sample in 0.15 M NaCI, $M_w$ was 390 kDa. Dextran sulfate, DSS ($M_W$ = 500 kDa, $M_n$ = 166 kDa, η (in 0.01 M NaCl) = 50 mL/g, 17% sulfate content, free $SO_4$ less than 0.5%) was produced by Fluka, Sweden (Reg. №. 61708061 A, Lot No. 438892/1). Surfactant free particles contains a negatively charged $SO_4^{2-}$ groups grafted to the surface of the polymer

particle with a diameters 0.21 um (product № 1-200, batch 1312.1; 0.91 um (product № 1-900; Batch № 20-326-9.3);3.0 um (product № S37223; Batch № 2272,1);5.0 um (Batch № 2434R 1*; product № S37227, Lot 51993A); 3.0 um CML/sulfonate latex contains a negatively charged $SO_2O^-$ groups grafted to the surface of the polymer particle (Batch № 636, product № 23-3000); 3.0 um aliphatic amino latex contains positively charged $NH_3^+$groups grafted to the surface of the polymer particle (Lot 466786, Batch № 271-4MD-1,1*) were produced by Invitrogen, USA.

*Preparation of the protein/polysaccharide and protein-polysaccharide-particles mixtures.* Most experiments were performed in the much diluted phosphate buffer (ionic strength, $I$= 0.002). To prepare molecularly dispersed solutions of SC, SA, or DSS with the required concentrations, phosphate buffer ($Na_2HPO_4/NaH_2PO_4$, pH 7.0, $I$=0.002) was gradually added to the weighed amount of biopolymer sample at 298 K, and stirred, first for 1 h at this temperature and then for 1 h at 318 K. The solutions of SC, SA, and DSS were then cooled to 296 K and stirred again for 1 h. The required pH value (7.0) was adjusted by addition of 0.1–0.5M NaOH or HCl. The resulting solutions were centrifuged at 60,000 *g* for 1 h at 296K, to remove insoluble particles. Concentrations of the solutions are determined by drying at 373K up to constant weight. The semidiluted W–SC–SA, W-SC-SA-DSS, and W-SC-DSS systems with required compositions were prepared by mixing solutions of each biopolymer at 296 K.

The suspensions of the particles were initially diluted by double bidistilled deionized water up to 2 wt% of particles. Two phase protein-polysaccharide systems containing 0.5 wt% particles were prepared by slow and careful mixing of the solution containing 2 wt% particles with two phase W-SC-SA-DSS system or single phase W-SC-DSS system in such way to obtain the final system containing 0.5 wt% particles. All experiments with W-SC-SA-DSS system were performed at the DSS/SC weight ratio, q equal to 0.14. After mixing for 1 h, two phase systems were kept at room temperature for 3 h. to separate the phases.

*Bright light microscopy* is used to visualized particles distributed in coexisting phases, using an Olympus BX51W1 fixed stage microscope equipped with a high resolution CCD-camera, (1000[1000 pixels, C-8800-21, Hamamatsu).

*Determination of the phase diagram*. The effect of the presence of particles on the isothermal phase diagrams demixed SC-SA systems was investigated using a methodology described elsewhere[19]. The weight DSS/SC ratio in the system, (q) was kept at 0.14. The threshold point was determined from the plot as the point where the line with the slope −1 is tangent to the binodal. The critical point of the system was defined as the point where the binodal intersects the rectilinear diameter, which is the line joining the centre of the tie lines. Symmetry coefficient $K_s$ was determined as a ratio of concentrations SC and SA at the critical point.

*Rheological measurements* were performed using a Physica Rheometer, type CSL2 500 A/G H/R, with a cone-plate geometry CP50-1/Ti ~diameter 5 cm, angle 0,993°, Anton Paar. The temperature was controlled at 23 °C by using a Peltier element. For each sample, flow curves were measured at increasing shear rate ~from 0.1 to 150 $s^{-1}$. The ramp mode was logarithmic and the time between two measurements was 30 s. Frequency sweeps ~0.1–200 rad/s were carried out as well for a strain of 3.0%, which was in the linear response regime. During the rheological measurements, all samples were covered with paraffin oil to avoid drying.

*Environment scanning electron microscopy (ESEM).* Micro structural investigation was performed with the environment scanning electron microscop**e** Philips XL30 ESEM FEG.

The instrument has the performance of a conventional SEM but has the additional advantage that practically any material can be examined in its natural state. The samples were freeze-fractured in freon and immediately placed in the ESEM. Relative humidity in the ESEM chamber (100%) was maintained using a Peltier stage. Such conditions were applied to minimize solvent loss and condensation, and control etching of the sample. Images were obtained within less than 5 minutes of the sample reaching the chamber. The ESEM images were recorded multiple times and on multiple samples in order to ensure reproducibility.

## RESULTS AND DISCUSSION

Cryo ESEM images of the two phase W-SC(6 wt%)-SA(0.29 wt%)-DSS system, and single phase W-SC(6 wt%)-DSS system shows no significant difference in their structure (Figure 1). In the both cases the main structural element visualized by ESEM is a network created jointly by SC and DSS. A small difference between these two images consists only in the presence of thickening for two phase W- SC(6 wt%)-SA(0,29 wt%)-DSS system. Figure 2 presents photography's of semidilute two phase W-SC(1.6 wt%)-SA(0.29 wt %)-DSS systems without particles (Fig 2 a ) and with 3 um particles (Figure 2 b), as well as microphotography's of the same system containing 3 um particles (Figure 2 c), and its SC enriched phase (Figure 2 d). One can see that addition of 0.5 wt% micro particles to demixed SC-SA system leads to their complete localization in SC enriched continuous phase. The same results were obtained for the same two phase system containing smaller (0.21 um and 0.91 um) and larger (5 um) particles; all the particles were distributed into the SC enriched phase. Use of the positively charged aliphatic amino latex (3 um) gave also the same results (data are not presented). Obviously charged micro spheres shows significant affinity to the SC enriched phase of the system, the main component of which, SC is polyampholyte containing negatively and positively charged functional groups. To visualize the structure of SC enriched phase containing particles, the samples were freezed, gold-coated, and studied by cryo ESEM.

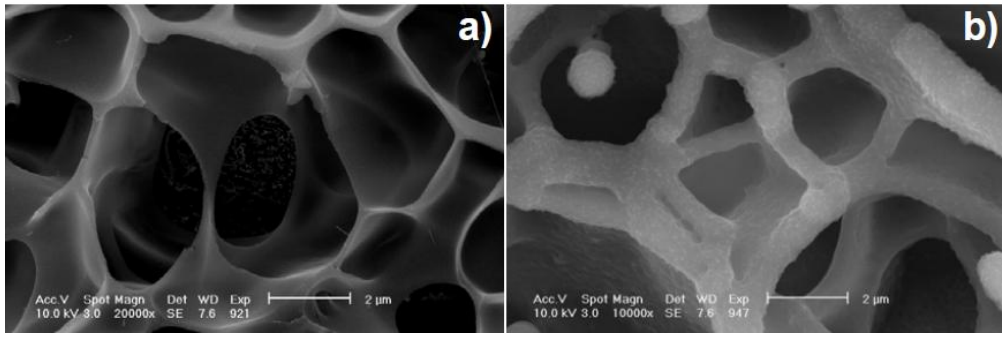


Figure 1. Typical ESEM images of the single-phase W-SC(6 wt %) -DSS (a) and two –phase W-SC(6 wt%)-SA(0.29 wt %) -DSS systems (b). DSS/SC weight ratio, q=0.14.

As is shown in Figure 3 all the particles distributed into SC enriched phase are incorporated in the SC-DSS network independently from their sizes, but the final structure of SC enriched phase is strongly determined by the sizes of the particles. Small particles with the

size (0.91 um) less the middle diameter of the network holes (2-3 um) are fixed on the one side of the network wall by means of plaque (Figure 3 a).

The binding of the particles takes place by formation of plaques connecting particles with the reactive charged groups of the SC-DSS network, indicated by arrow in Fig 3 a. The same ESEM images were obtained for SC enriched phase the same system containing smaller (0.21 um) particles. (Data are not shown).

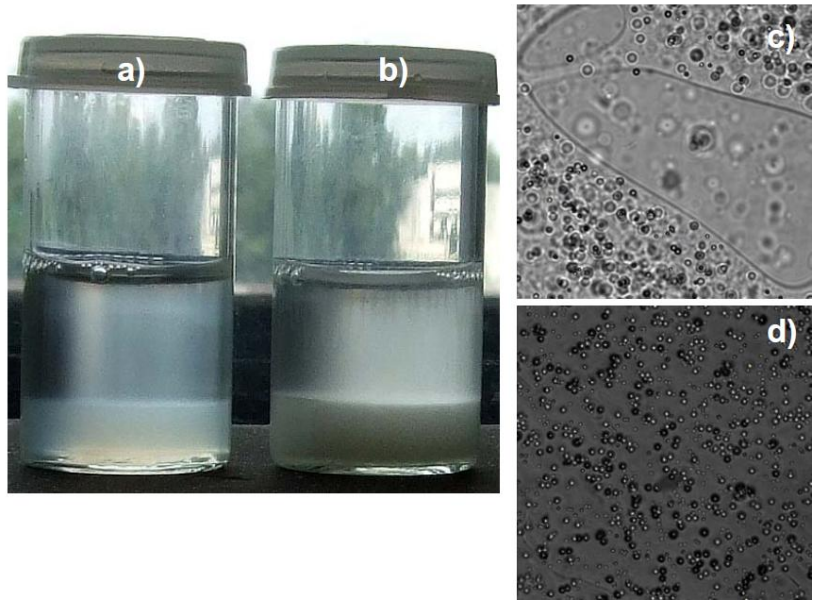


Figure 2. Photography's of semidilute two phase W-SC(1.6 wt%)-SA(0.29 wt %)-DSS systems without particles ( a ) and with 3 um particles of sulfate latex( b), as well as microphotography's of the same system containing 3 um particles (c), and its SC enriched phase (d). DSS/SC weight ratio, q=0.14.

In the later system, formation of plaques connecting particles with the active sites of the SC-DSS network may be seen especially distinctly. Another type of the structure was observed for the relatively large (5 um) particles dispersed into the demixed system (Figure 3 c). These particles has diameter larger than sizes of the holes inside the network. Their incorporation into the network leads to significant damage and destructions of the latter as it can be seen in Figure 3 c.

The most interesting structure was observed in the case of the system incorporating particles having 3 um in diameter. These particles are similar in size with that of the network holes (2-3 um). As can be seen in Figure 3 b the particles of CLM-Sfn latex build ideally into the network without damage of the later. The same result was obtained for 3 um sulfate latex.

Incorporation of these particles inside the network has one interesting feature. Small structural elements of these charged particles inside the network develop perfect orientation from the center in to direction of periphery. Width of these small structural elements (about 10 nm) is comparable with the distance between sulfate groups in polysaccharide molecules[20]. Therefore we can reliably assume that incorporation of the particles inside the network leads to the total polarization of their structure elements including negatively charged sulfate groups.

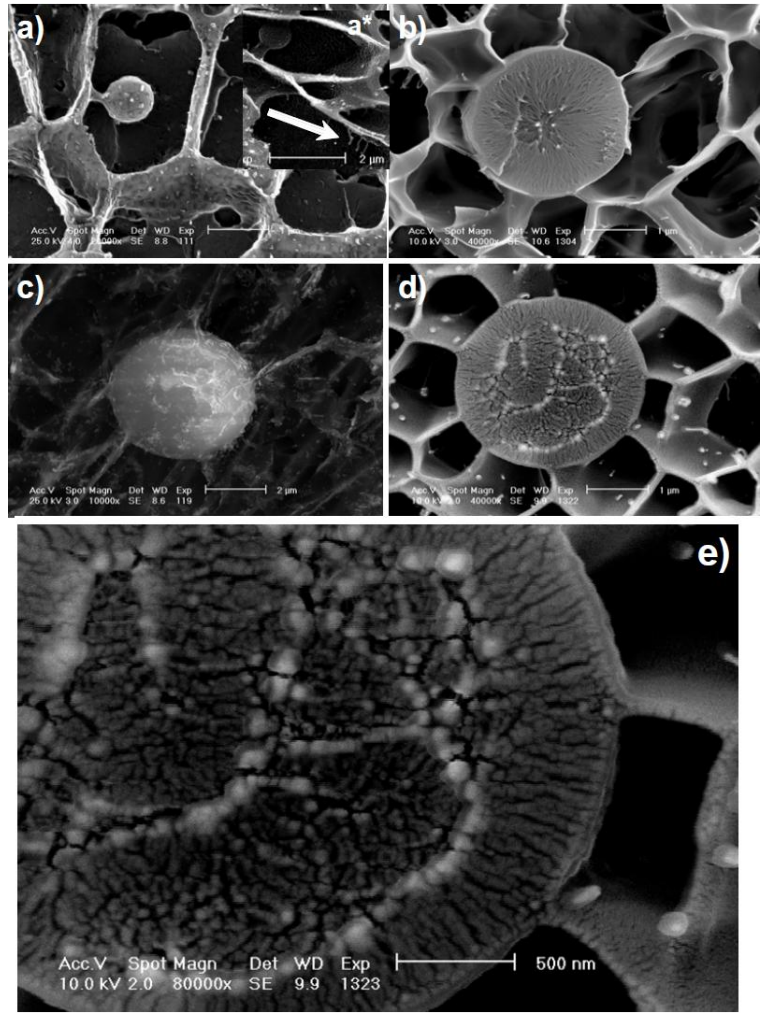


Figure 3. ESEM images of SC enriched phase W- SC(1.6 wt%)-SA(0.29 wt%)-DSS system in the presence of 0.5% particles. (a,c) - sulfate latex particles with a diameters 0.91 um and 5.0 um correspondingly; carboxylate modified sulfonate latex with a diameter 3 um (b), and 3.0 um aliphatic amino latex (d,e). q=0.14. ESEM images of W-SC (1.6 wt%)-SA (0.29 wt%) system in the presence of 3.0 um aliphatic amino latex with a diameter 3 um (f,g).

The particles having the size 3 um and containing positively charged amino groups are also perfectly incorporated inside the network (Figure 3 d). Microphotography of SC enriched phase containing these particles shows formation of two types of structure inside the particles in the process of their interaction with SC enriched phase. The first type contains the structural elements which oriented from center to periphery. Note, that the structure of the particles qualitatively is the same as before incorporation in the biopolymer mixture.

However, higher orientation of the second type of structural elements in the SC enriched phase W- SC (1.6 wt%)-SA (0.29 wt%)-DSS system in the presence of 0.5% particles shown in Figure 3 (d, e) compared with their orientation in the absence of DSS (data are not shown) may be the result of interaction and incorporation of the particles into the SC-DSS network. It is necessary to say that caseins adsorbs strongly at the surface of latex particles.

It has been shown that there is typically a saturation surface coverage of protein of 1–3 mg/m2, and the particles in the protein solution then act not as charged bare latices but as sterically stabilized protein-coated particles. However, in the presence of interacting polysaccharides like dextran sulfate, the adsorbed layer structure is more complex.

This type of the structure was detected close to periphery of the particles, far from the center. From the producer information we know that just amino groups of the latex placed at the periphery of the particles. These groups has a greater freedom to rotate, therefore in a free unbound state they does not oriented along the line from the center of the particle to periphery. Thus, their orientation shown in Figure 3 (d,e) may be the result of interaction and incorporation of the particles into the SC-DSS network.The second type of the structure include weakly oriented structural elements, and it placed in the central part of the particles. It is important to note that incorporation of 3 um particles into the SC enriched phase are not exceptional phenomenon which is observed only in the presence of DSS in W-SC-SA system. The use of the same particles in the SC enriched phase containing 0.06 wt% lambda carrageenan instead of DSS leads to the same result (Figure 4).

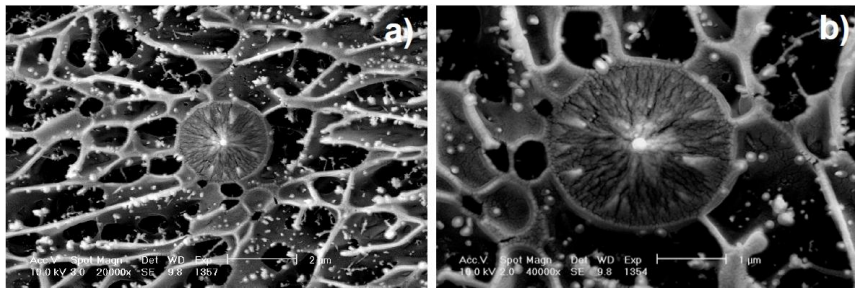


Figure 4. ESEM images of SC enriched phase W-SC(1.6wt%)-SA(0.29wt%)-lambda carrageenan systems in the presence of 0.5% 3 um carboxylate modified sulfonate latex. Lambda carrageenan/SC weight ratio, q=0.06.

Since the size of the charged particles has a strong effect on the structure of W- SC-SA-DSS system, the important question is arise; whether the presence of low-volume fractions of nano- and microparticles effect the phase separation?

In order to quantify the effect of the particles on phase equilibrium in semi diluted demixed SC-SA system, the isothermal phase diagrams of the system were determined at 25$^{o}$C in the presence of 0.21 um, 0.9 um, 3.0 um and 5.0 um particles, plotted in the classical triangular representation (Figure 5, curves 2, 3), and compared with that obtained in the absence of particles (Fig 5, curve 1). The phase separation in all cases has a segregative character with preferential concentrating of SC and SA in different phases. The degree of compatibility was valuated by comparison position of the binodal line, the critical, and threshold points. The phase diagram of the demixed system, without particles is characterized by a low total concentration of biopolymers at the critical and threshold points ($C_t^{cr}$ = 10.6 g/L, $C_t$*= 6.2 g/L), and a relatively strong asymmetry ($K_s$=4.9). The presence of small particles with the sizes 0.21 um and 0.91 um do not effect appreciably on the position of the binodal line, and the critical and threshold points. We did not observe any difference in thermodynamic behavior of these systems, at least at concentration particles in the mixtures studied (0.5 %). In the contrast to small particles, the presence of 3 um CML sulfonate or sulfate particles in the system affects the phase separation, significantly increasing the

concentration range corresponding to two phase state. The total concentration of biopolymers at the threshold point decreases to 5.2 g/L. It seems that degree of thermodynamic compatibility of SC with SA after an addition of the 3 um particles is the smallest known for biopolymer mixtures. The dominant mechanism responsible for decrease thermodynamic compatibility in such system may be intensification of self association of SC in the SC enriched phase due to perfect build 3 um particles into the holes of the network of SC enriched phase (2-3 um in diameter), and reinforcement of this network. The phase diagram of W- SC-SA-DSS system in the presence of 3 um particles is more asymmetric ( Ks =10) compare with that free from the particles. This is not unexpected phenomenon, because it is known that the stronger association of the polymer 1 in the polymer 1-polymer 2-solvent system than higher weight ratio (polymer 1/polymer 2) at the critical point, in other words, than higher asymmetry of the phase diagram obtained.

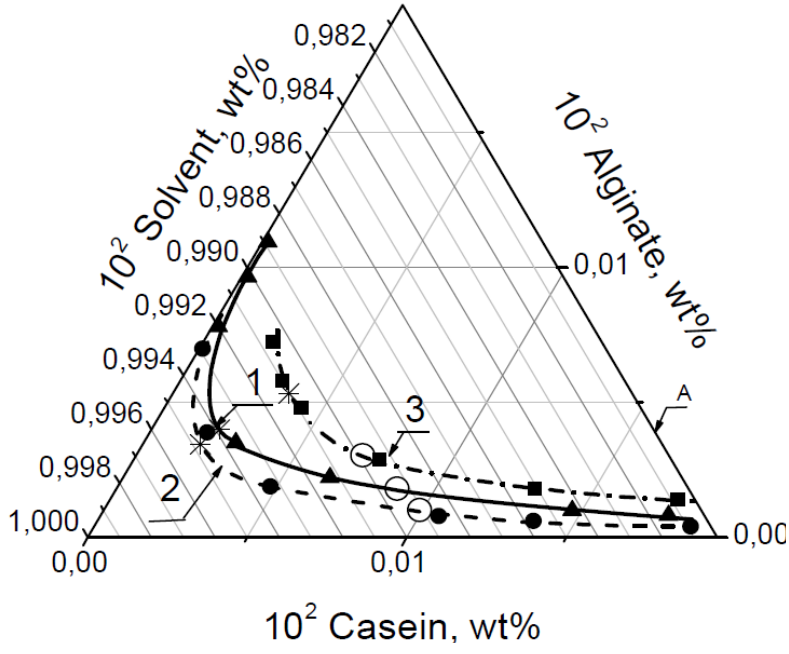


Figure 5. The effect of the presence of micro particles on isothermal phase diagrams of water- SC-SA-DSS system. 296 K. DSS/SC weight ratio, q=0.14. Curves 1-3-binodal lines. Sizes of the particles: curve 1, describe the binodal line for the system without particles, and in the presence 0, 91 um sulfate latex, curve 2,-the binodal line in the presence of 3.0 um sulfate latex, curve 3,- the binodal line in the presence of 5.0 um CML sulfonate latex. 296 K. ○ critical point; *- threshold point.

Just opposite effect was observed in the case of the presence 5 um particles in the demixed SC-SA system (Figure 5, curve 3). As it was described above (see Figure 3 c), these particles induce the partial damage of the network presented in the SC enriched phase. The main result of this damage is increase thermodynamic compatibility of SC with SA, and decrease asymmetry of the phase diagram. The total concentrations of the biopolymers (SC and SA) at the threshold point increases from 6.2 g/l to 9.0 g/L, and asymmetry coefficient, Ks decreases from 5 to 2.34. The main conclusion from these results is that the ratio of the particle size to the middle size of the network holes, (R) plays the key role in the phase equilibrium of the demixed biopolymer system. If this value is close to 1, thermodynamic

compatibility of biopolymers decreases. If R>1, it leads to increase in compatibility. At R values less or equal 0.3, the presence of the particles do not effect noticeably neither position of the binodal line, neither the main parameters of the phase diagram. We established that decrease compatibility of the biopolymers in the presence of 3 um particles become more significant among the next order:CML latex < aliphatic amino latex< sulfate latex ≅CML/sulfonate latex.

Since addition of particles in the separated W-SC-SA system leads to their incorporation into the SC enriched phase, the dynamic modules and the viscosity of the casein-DSS systems were measured without the presence and with the presence of 0.91 um, 3 um and 5 um particles Figures 6 shows the dynamic modules in dependence on the frequency for the water –SC (10 wt%)-DSS (1.4 wt%) system before and after addition of the particles (Figure 6 a) and the flow curves of these systems (Figure 6 b).The presence of small micro particles in the system (0.21 um and 0.91 um) do not effect appreciably on the dynamic modules and viscosity, whereas the particles having diameter 3 um increase appreciably the modules and viscosity of the system. On the contrary, the larger particles (5 um) decrease the dynamic modules and viscosity of the SC-DSS system. Such behaviour is an agreement with the data of the structural study SC-DSS systems after addition of the particles. (Figure 2). Incorporation of the 3 um particles into the SC-DSS network holes probably make this network more solid increasing the modules (Figure 6 a) and viscosity (Figure 6 b) of the system. At frequency values equal to 0.13 rad/s, G' and G'' values are more than 1.4 and 1.6 times, respectively, higher compared with those of the emulsion without particles. The particles with the size larger than the size of the network holes disrupt partially network decreasing the modules and viscosity of the system.

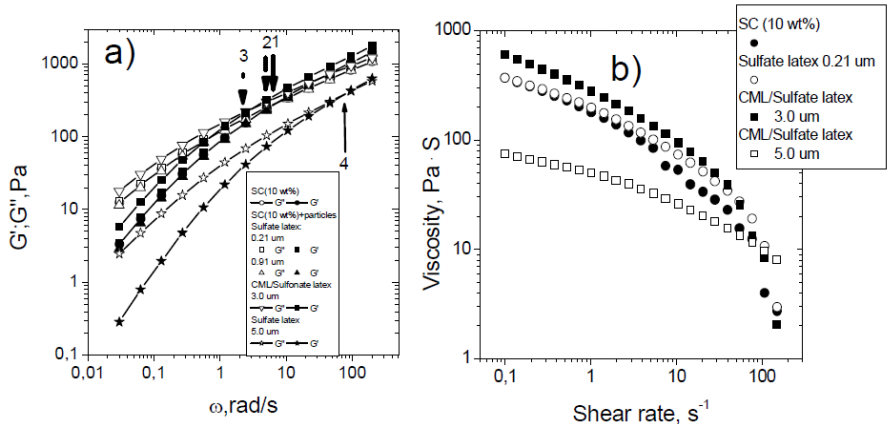


Figure 6. The dynamic modules in dependence on the frequency of the aqueous sodium caseinate (10.0 wt%)-DSS systems at q=0.14, obtained in the absence and in the presence of 0.5 % particles at. 296 K. ( a ) , and the flow viscosity of the same systems (b). 296 K.

It is interesting to note that crossing G' and G" in the case of the system containing 3 um particles takes places at lower frequency than for the system without particles, and crossing G' and G" for the system containing 5 um particles observe at higher frequency values compared with that of the system do not containing particles. It means that the time life of the network is longer in the case of the demixed system containing 3 um particles. For the rheological investigations of the phase separated SC-SA system containing particles, we

separated by centrifugation the two-phase SC (1.6 wt%)-SA (0.4 wt%)-DSS (0.22 wt %) system (point A on the phase diagram, Figure 5), containing 3 um particles. The emulsions W-SC (0.44 wt%)-SA ( 0.67 wt%), W-SC (0.73 wt%)-SA (0.59 wt%), W-SC (1.02 wt%) – SA (0.52 wt%), and W-SC (1.6 wt%)-SA (o.4 wt%) containing 15% vol, 25% vol, 35% vol and 50% vol alginate-enriched dispersed phase and placed on the phase diagram along the same tiel line connecting compositions of the coexisting phases were then characterized through their viscoelastic behaviour. First, the mixture was presheared at 500/s for 900 s, in order to wipe out any previous mixing history.

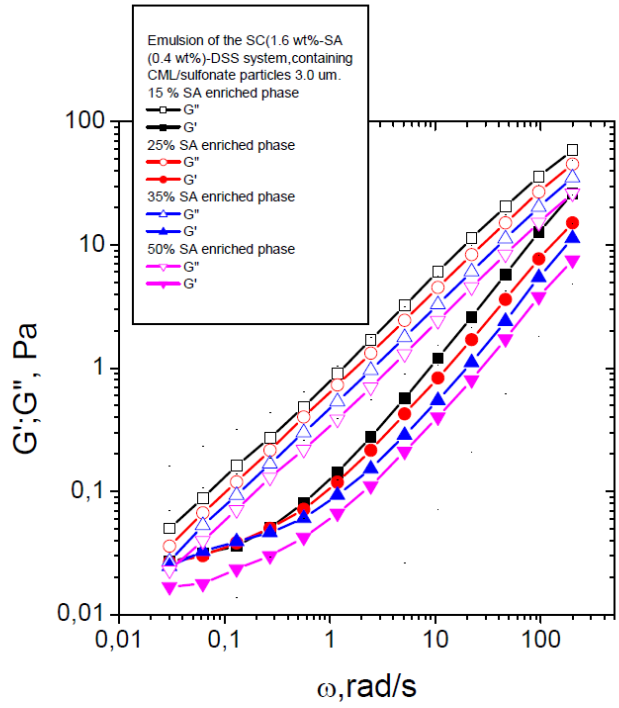


Figure 7. The dynamic modules in dependence on the frequency of the aqueous SC(1.6 wt%)-SA(0.4 wt%)-DSS(0.22 wt%) system at q=0.14 and 296 K.

The sample was allowed to relax for 60 s, leaving enough time for full relaxation of the deformed droplets. Finally, a dynamical spectrum was measured in order to characterize the state of the emulsion. The obtained results are presented in Figure 7. The spectrum of the emulsion shows the existence of a low frequency plateau. Such a plateau would be the signature of a weak (small G' values) network structure at the emulsion scale (lower frequencies = larger structure scale).

## CONCLUSION

The use of nano and micro particles to affect the phase separation and morphology in (bio) polymer mixtures is a topic of great scientific and technological interest. We have shown that if protein enriched phase of a two phase aqueous protein-polysaccharide system

form a weak network during interaction of the protein with sulfate polysaccharide, then the presence of small fraction of the charged 3 um particles in such system lead to significant decrease compatibility of biopolymers and increase the viscoelastic properties of the emulsion. The effect of the presence of particles is especially significant in the case of use hydrophobic CML/sulfonate and sulfate micro particles. The dominant mechanism responsible for decrease thermodynamic compatibility in such system is formation of the large charge microparticle (3 um) -SC/DSS associates in SC enriched phase that leads to decrease their compatibility with alginate due to the effect of the excluded volume interactions between SA and microparticle-DS+SC associates.. Hence, optimizing the sizes and degree of hydrophobisity of particles will be possible affect on the phase separation of biopolymer systems.

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*Chapter 11*

# ADSORPTION BEHAVIOUR OF 5-METHYLRESORCINOL AND ITS MIXTURES WITH LYSOZYME AT AIR-WATER INTERFACE

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## ABSTRACT

The adsorption kinetics of 5-methylresorcinol (MR) at water-air interface after 2 and 24 hours incubation of MR solutions in 0.05M phosphate buffer with pH 7.4 at $25^{O}$C was studied using a dynamic drop tensiometry via drop shape analysis techniques. The influence of methylresorcinol on adsorption of a model protein (hen egg lysozyme) was studied at fixed protein concentration 3.4 $10^{-6}$ M and varying methylresorcinol concentration.

## INTRODUCTION

Nowadays there is a strong trend towards investigation of structure and functions of microbial low molecular extra-cellular metabolites, represented in a number of bacteria and yeast cells by alkylhydroxybenzenes (AHB). Alkylresorcinols in particular, inducing transition of microbial cells into a hypo-metabolic (anabiotic) state [Bespalov et al., 20000; Kolpakov et al., 2000], are capable also to affect the functional activity, operational stability and substrate specificity of some enzymes [Petrovskii et al., 2009; Martirosova et al., 2004]. Another direction of using AHB, having a great practical importance, is modification of enzymes (lysozyme, for example) to increase its resistance to action of microorganisms with maintenance of its activity. Lysozyme is widely used both in medicine when treating infections diseases and in the food industry to prevent bacterial infection of products. In this connection, acquisition of resistance to lysozyme by microorganisms is becoming an

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important issue [Deckers et al., 2004; Radwan et al., 1994; Lesnierowski et al., 2004]. AHBs realize modifying functions through weak non-specific interaction with enzymes and wide variety of biopolymers of a bacterial cell [El'-Registan et al., 2006; El'-Registan et al., 2005]. One of the homologous – 5-methylresorcinol exhibited the most stimulating effect on enzymatic activity of some hydrolases [Martirosova, 2007]. 5-Methylresorcinol has been noted to stimulate the lysozyme activity within the range of concentrations $10^{-7} – 10^{-3}$ M up to 200% when peptidoglycane from the *Micrococcus luteus* is used as a substrate, and up to 470% when a nonspecific substrates (colloid chitin, *Saccharomyces cerevisiae* cells) are used [Petrovskii et al., 2009]. Non-specific influence of these auto-regulators on enzymatic proteins is associated with the chemical structure of AHB and the type of their interplay with protein molecules [Martirosova et al., 2004; Nikolaev et al., 2008]. Also correlation between the alteration of the catalytic activity of lysozyme under the influence of MR and its destabilizing effect on the native conformation of the enzyme was established [Plashchina et al., 2009]. This effect was exhibited in the drop of excess Gibbs's denaturation free energy ($\Delta_d G$), as well as in the rise of difference of the heat capacity between a native and denatured state ($\Delta_d C_p$) with the increase of the modifier concentration. This testifies to destabilization of the native conformation of the protein and a predominant interaction of MR with its denatured form. The activity and thermostability of the lysozyme has been shown to depend on the MR concentration and the incubation time of their combined solutions [Martirosova et al., 2010a]. Increase in the time of enzyme modification up to 120 hours results in a significant drop of an activating effect of MR. The obtained data provide evidence that under a long-term exposure of lysozyme in a combined solution with MR, hydrophobic hydration of the protein grows and the cooperative unit of a conformational transition alters. Effect of MR on the alteration of kinetic parameters ($K_m$ and $V_{max}$) of lysozyme in the hydrolysis reaction of colloid chitin indicated on activation of an uncompetitive type [Martirosova et al., 2010b] and also is confirmed for other model enzymes (tripsin, β-amylase) whose modification by MR led to an uncompetitive activation [Nikolaev et al., 2008; Martirosova, 2007]. The effect of 5-methylresorcinol on structure, equilibrium fluctuations and nonspecific activity of lysozyme have been studied in the broad range of concentrations [Krupyanskii et al., 2011]. It is shown that 5-methylresorcinol interact with the surface of lysozyme molecule directly, not via hydrogen bonds. This leads to an increase in the amplitude of equilibrium fluctuations which allows him to be effective activator. Influence of the MR concentration on thermodynamic parameters of its interaction with hen egg white lysozyme in 0.05M PBS at pH 7.4 and 37$^o$C has been investigated by ITC [Martirosova, 2011]. It is shown that interaction between lysozyme and MR has an exothermic character and is characterized by low value of binding constant (26.6 $M^{-1}$ for 37$^o$C). To record a thermal effect of the MR dilution, we performed a single titrant injection into the buffer solution. The resulting isotherm demonstrates that an exothermal mode of MR interaction with the buffer is replaced by an endothermal one as the ligand concentration grows. The transition region corresponds to the MR concentration of 20 mM (2.5 mg/mL), which is likely to be due to the process of self- association of 5-methylresorcinol molecules. We propose that MR in own aqueous solutions and mixed solutions with lysozyme behaves similar to nonionic surfactants due to the diphylic character and ability to self-organization. The data on surface activity of MR in literature are not found. The aim of this paper is to investigate the dynamic of adsorption of MR from aqueous solutions at the air/water interface depend on concentration, storage time at 25$^O$C and the presence of hen egg white lysozyme.

## MATERIALS AND METHODS

A sample of hen egg white lysozyme (Sigma, USA) with activity 20,000 U/mg and molecular mass 14,445 Da was used.

Alkyl-substituted hydroxybenzenes, 5-Methylresorcinol (5-Methylbenzene-1,3-diol) (Sigma, USA) with molecular mass 124,14 g/mol (anhydr) was taken.

All reagents for PBS preparation in Milli-Q water were of analytical grade.

### Surface Tension Measurement

Dynamic surface tension was measured with a Tracker tensiometer (ITC, France), connected to thermostatic bath to maintain the temperature constant at $25^{O}$C during the measurements. The principle of tensiometer is to determine the surface tension of the studied solution from the axisymmetric shape of a rising bubble analysis [Loglio et al., 2001]. Due to the active control loop, the instrument allows long-time experiments with a constant drop/bubble volume or surface area.

Surface tension, σ (mN/m), was measured in 7 mL samples at constant lysozyme (0.05 mg/mL) and varying 5-methylresorcinol (0.16-128.2 mM) concentrations and their mixed solutions in 0.05M PBS, pH 7.4 at $25^{O}$C.

The solutions of pure MR were taken for surface tension measurement after 2 or 24 hours of storage at $25^{O}$C in darkness. The mixed solutions LYS-MR were prepared with using of lysozyme and MR after 24 hours incubated separately. After mixing of these solutions 1:1 they were stored 3 hours at $25^{O}$C away from light. The dynamic surface tension was measured over 30 000 s to guarantee steady-state of the adsorption layer. A $t\rightarrow\infty$ asymptotic extrapolation was used to find the steady-state surface tension values. Standard deviations were always less, than 0.5 mN/m, and duplicate measurements were made for each MR concentration. Finally phosphate buffer were confirmed not to present surface activity by measuring separately the surface tension of a PBS buffer solution, obtaining values practically equal to those of pure water.

From the kinetic curves (surface tension versus time) for the solutions of different composition, the steady-state surface tension isotherms (surface tension versus concentration) were obtained. As the concentration of the protein in the mixture was fixed, the abscissa in all graphs represents the concentration of the surfactant in the mixed solution. From surface tension data the MR critical concentration of self-organization (micelle-formation) in the absence and in presence of lysozyme were estimated. For this purpose surface tension values were fitted with the logarithm of the MR concentration.

## RESULTS AND DISCUSSION

### Adsorption of Methylresorcinol

Surface tension is a phenomenon which arises due to the existence of intermolecular forces in solution. The molecules at the interface experience an inward pull from bulk

molecules due to the intermolecular forces which is measured by the surface tension. In order to avoid the unfavorable energetic interaction of its nonpolar part with water, a surfactant molecule prefers to partition to the air–water interface, resulting in the reduction of the surface tension. A dynamic equilibrium always exists among the surfactant monomers at the interface with the species in the bulk solution below CMC. Beyond CMC, surfactant monomers at the interface equilibrate with micelles in the bulk medium [Dash, Misra, 2011]. In figure 1. dynamic curves of MR adsorption at air/water interface from 0.05M PBS (pH 7.4) solutions of varying concentration (0.16-128 mM) are presented. The data relates to time of storage of MR solutions 2 and 24 hours at 25$^{O}$C in darkness (Figure 1a and Figure 1b, respectively). One can see that MR in aqueous media exhibits behavior similar to that of surfactants which reduce the surface tension of water at the interface with air.

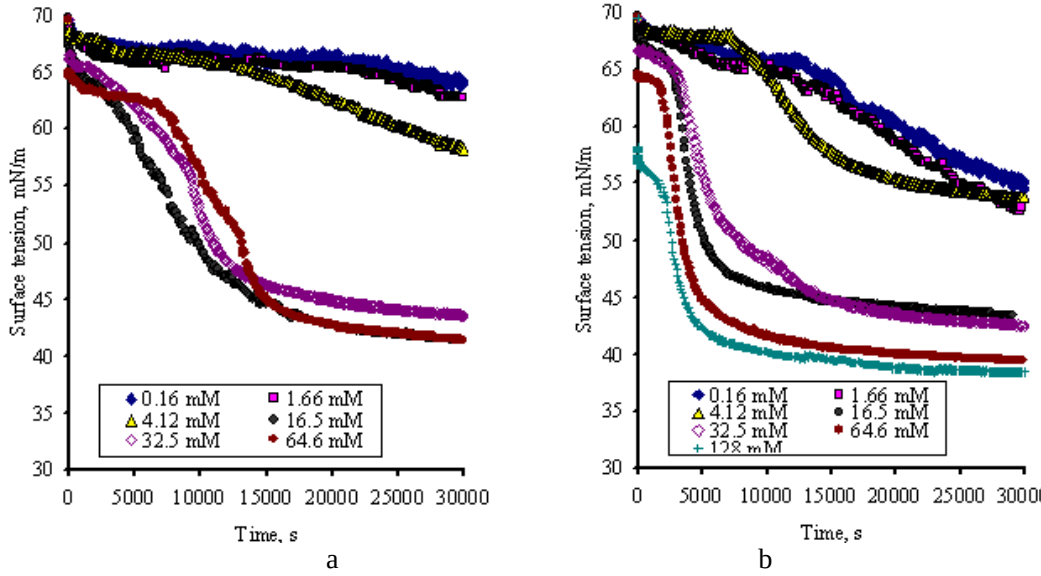


Figure 1. Dynamic surface tension of MR concentrations 0.16-128 M after 2 h (a) and 24 h (b) incubation and storage t 25$^{O}$C in darkness.

The surface activity of MR is a function of concentration and previous storage time of solutions. The form of dynamic curves changes with increasing storage time of solutions from poorly reproducible multistep (Figure 1a) to more simple, well reproducible (Figure 1b).

Possibly this change manifestoes the change of the MR structure in water solution.The existence of lag- or induction period which is typical for all dynamic curves of MR adsorption means that adsorption process is diffusion-limited [Fainerman et al., 1994].

The value of induction period drops with increasing of MR concentration up to 0.0165 M, and then does not change (Figure 2). Early on the base of ITC data [Martirosova, 2011] we proposed that this point corresponds to transition of MR from molecular dispersed state to associative one. The surface tension isotherm of MR adsorption obtained at 2 and 24 hours of time storage is shown in Figure 3. In agreement with expectation isotherm has a form typical for surfactant.

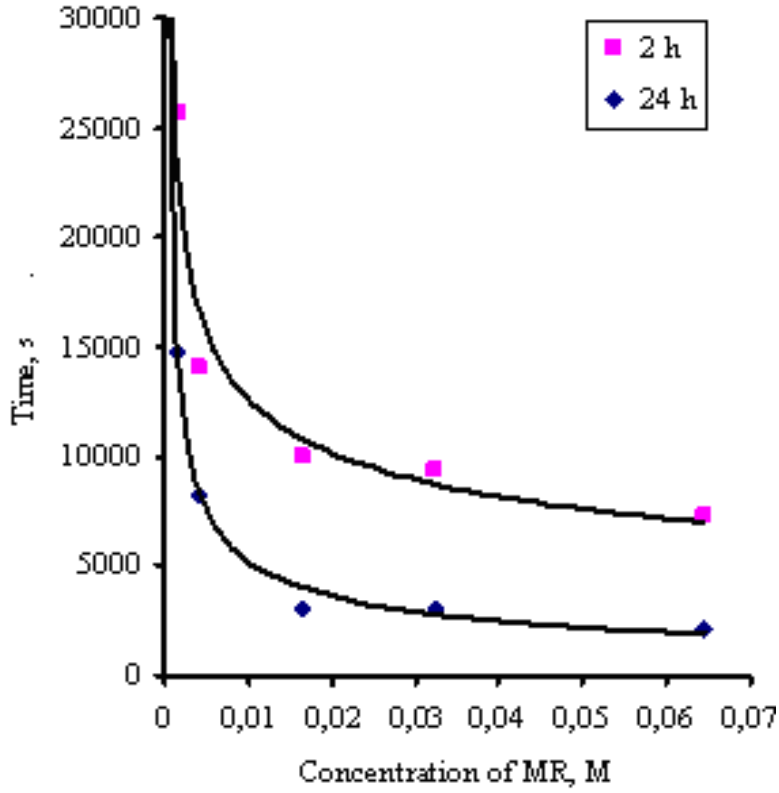


Figure 2. Induction period value of dynamic curves of MR adsorption at air/water interface as function of MR concentration. Temperature 25$^{O}$C; storage time of MR solution before measurement 2 and 24 hours.

Dehydroresorcinols, as is known, may exist in three tautomeric forms in water solution (di-enol, keto-enol and keto) [Durairaj, 2005]. This compound is also known as cyclic beta-diketone and has been shown to exist to 95% in the keto-enol (or mono-enol) form in aqueous solution. The $pK_a$ value for dehydroresorcinol is about 4.8 and, therefore it is considered an acid and almost as strong as acetic acid. The hydroxyl groups of phenolic derivatives are more acidic than that of aliphatic alcohols, and hence they can form stronger hydrogen bonds as donors with suitable bonding acceptors (as in our case at pH 7.4). In certain cases, at acidic pH values, the oxygen atom of hydroxyl group of phenol can also behave as an effective hydrogen–bond acceptor. Generally, O − H···O hydrogen-bonding interactions between hydroxyl groups are strong and highly directional. It is reasonable to expect that the presence of two O−H groups in MR would impart in intrinsic molecular and supramolecular dimensions to MR. Apart of O − H···O interactions, a few other intermolecular interactions such as C−H···O, O−H···π and O−H···N are also important. However these interactions are weak in nature compared to conventional hydrogen bonds [Sarmat, Baruah, 2007]. Owing to hydrogen bonding dihydroresorcinols are able to self-assembly in aqueous solutions. Obviously the contact times 2 hours are too short to permit the attainment of a steady state. We choose the storage time of 24 hours for further experiments.

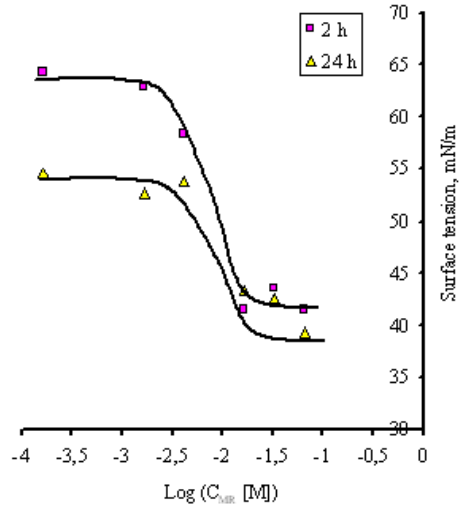


Figure 3. Surface tension isothermes for MR at 25$^{O}$C and times incubation: 2 and 24 hours.

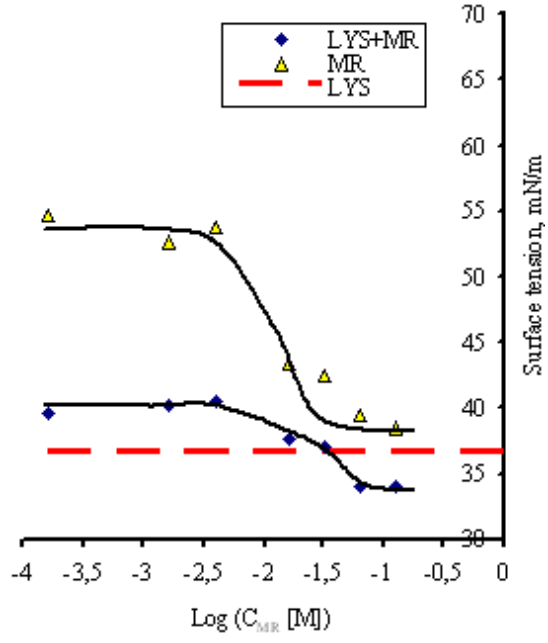


Figure 4. Surface tension isotherms for MR alone and in mixed solution with lysozyme at constant lysozyme concentration (3.4 $10^{-6}$ M) at 25$^{O}$C.

At steady -state a constant fraction of surfactant molecules stays at the interface and so an area per molecule can be calculated from the adsorption density at the air/water interface according [Dash, Misra, 2011].

The surface tension data are fitted to the Gibbs adsorption equation to calculate the amount of surfactant adsorbed per unit area of air/water interface. The Gibbs adsorption equation [30] may be written as:

$$d\sigma = -\Gamma\, d\mu = -\Gamma RTd\ln C \quad (1)$$

where dσ, Γ, dμ, R, T, and C are the change in the surface tension in the solution, the adsorption density of the surfactant, the change in the chemical potential of the surfactant, universal gas constant, absolute temperature, and the concentration of the surfactant in aqueous solution, respectively. Since the concentrations of the surfactant solutions are dilute the activity is replaced by concentration. Eq. (1) can be written as

$$\Gamma = -1/RT(d\sigma/d\ln C) \quad (2)$$

Maximum adsorption density is calculated by limiting the concentration in the above equation to CMC of the surfactant. Hence Eq. (2) can be expressed as

$$\Gamma_{max} = -1/(2.303RT)\ \text{limit}_{\ C\rightarrow CMC}\ (d\sigma/d\log C)_T \quad (3)$$

The minimum area per molecule ($A_{min}$) in $Å^2$ can be calculated from

$$A_{\ max} = 10^{20}/N_A\,\Gamma_{max} \quad (4)$$

where $N_A$ is the Avogadro number. The values of $\Gamma_{max}$ and $A_{min}$ are given in Table 1. The standard free energy of adsorption is obtained from

$$\Delta G_{ad}{}^{o} = \Delta G_m - (\Pi_{cmc}/\Gamma_{max}) \quad (5)$$

where $\Delta G_m$ is the standard free energy change of micellization

$$\Delta G_m{}^{\ o} = RTd\ln C_{cmc} \quad (6)$$

and $\Pi_{cmc} = \sigma_{\ water} - \sigma_{\ cmc}$ the values are given in Table 1.

**Table 1. CMC and area per minimum from surface tension data at 25$^{O}$C**

| Sample | CMC mM | $\Pi_{cmc}$ (mN/m) | $\Gamma_{max} \times 10^6$ (mol m$^{-2}$) | $A_{min}$ ($Å^2$) | $-\Delta G_m$ (kJ mol$^{-1}$) | $-\Delta G_{ad}$ (kJ mol$^{-1}$) |
|---|---|---|---|---|---|---|
| MR | 30.2 | 30.8 | 3.00 | 55.4 | 8.67 | 18.9 |
| MR+LYS | 64.6 | 35.5 | 0.94 | 117.0 | 6.79 | 44.5 |
| CTAB* | 0.90 | 37.0 | 2.90 | 57.0 | 27.8 | 40.0 |
| SDS* | 8.5 | 36.5 | 2.60 | 63.0 | 22.1 | 35.2 |

*taken from [Dash, Misra, 2011] for the temperature 30$^{O}$C.

Comparative analysis of obtained parameters for MR with those for typical surfactants (CTAB and SDS) [Dash, Misra, 2011] shows that some of them are close to each other, for example, $\Gamma_{max}$ and $A_{min}$. At the same time, it is evident that MR is less surface active due to less negative values of such parameters as $\Pi_{cmc}$, $\Delta G_m$ and $\Delta G_{ad}$. Its micelle is less stable.

## Adsorption of Methylresorcinol in the Presence of Lysozyme

Several authors reported changes in adsorption kinetics for the mixtures of proteins and surfactants [Alahverdjieva et al., 2008; Maldonado-Valderrama et al., 2007; Wu et al., 2006]. For protein/ non-ionic surfactant mixtures there is a competitive adsorption [Alahverdjieva et al., 2008] from the very first addition of surfactant which increases with the surfactant concentration at a fixed protein contents and leads to a gradual displacement of the protein from the interface. From a certain surfactant concentration on the two isotherms, the mixture and the pure surfactant, overlap indicating a surface layer covered essentially by free surfactant molecules. The dynamic surface tension measurements performed in phosphate buffer solutions (pH 7.4, $I$ = 0.05 M) at constant lysozyme concentration, 3.4 $10^{-6}$ M, and varying concentrations of MR, show that the LYS-MR mixture is more surface active than the pure MR in concentration < $CMC_{mix}$. Moreover, adsorption rate is enhanced with respect to that of pure MR at the same concentration (Figure 4, 5). The Lys-MR mixtures are slightly more surface active than its both components alone at MR concentration > $CMC_{mix}$. (64.6 mM).

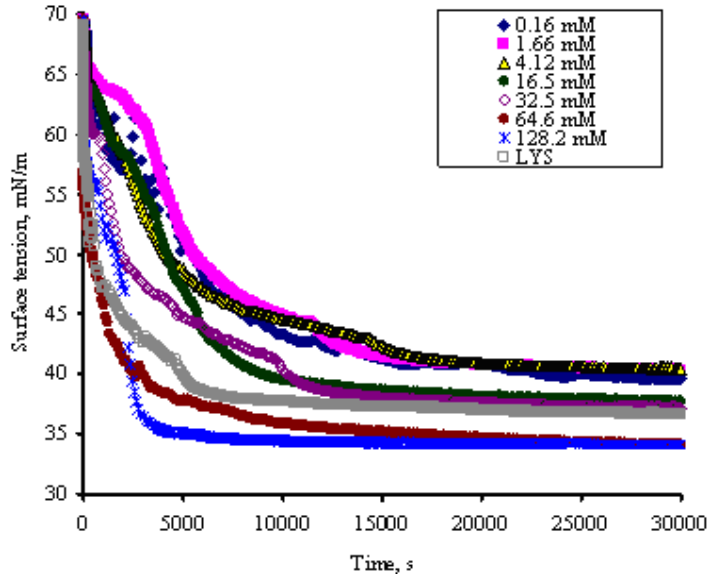


Figure 5. Dynamic curves of adsorption of MR in mixture with lysozyme at air/water interface at 25$^{O}$C; MR concentrations 0.16-128.2 mM and constant lysozyme concentration 3.4 $10^{-6}$ M.

The interaction in the mixture may be of either synergistic or antagonistic. The synergism leads to the enhancement of the surface activity of the mixture which in turn lowers the CMC of the mixed system compared to the individual components. Antagonism is a result of competition of surfactants in adsorption process due to difference in their hydrophobicity and possible unfavorable sterical effects of their structure. Consequently, micelle formation takes place at higher concentrations.

As concerning the effect of MR on the surface activity of lysozyme, it is seen from figure 4 that MR decreases the surface activity of lysozyme at concentration < $CMC_{mix}$ and

enhances it at concentration > $CMC_{mix}$ making lysozyme more hydrophilic or hydrophobic, correspondingly. Both effects are rather low.

## CONCLUSION

Adsorption of MR was shown to be mixed-diffusion barrier controlled. MR behaves similar nonionic surfactant. MR was also shown to change thermodynamic affinity of lysozyme to the solvent with the resulting the protein being slightly less or more surface active depending on MR concentration. Under used conditions MR and lysozyme can compete in adsorption process at air/water interface.

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*Chapter 12*

# NEW PROBIOTIC PRODUCT FOR PREVENTION AND TREATMENT OF ENTERITIS AND RESPIRATORY DISEASES OF FARM STOCK

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## ABSTRACT

Application prospects of sporulating bacterial strain *Bacillus* subtilis for elaboration of new probiotic used in prevention and treatment of respiratory diseases in farm stock were demonstrated. Pilot-plant technology of Bacinil manufacturing and application was developed. Prophylactic and therapeutic efficiency of Bacinil in cases of enteritis and respiratory diseases of farm animals was established. Bacinil was registered by Vetbiopharm Council and entered into the list of veterinary drugs authorized for use in Belarus.

**Keywords:** Sporulating bacteria, Bacillus subtilis, fermentation, biological control, animal pathogens, biotechnology, probiotic

One of the problems of modern dairy and meat stock breeding is increased incidence of infectious diseases caused by facultatively pathogenic bacteria showing a broad spectrum of virulence (enterotoxigenecity, adhesive capacity, haemolytic activity, drug resistance). Latest reports indicate general epizootic significance of gastrointestinal pathologies in young animals manifested as diarrhea syndrome [1, 2]. The reasons inducing gastroenteritis cases in animals may be defects in rearing conditions, malnutrition, disbalance in gut microbiota, immune deficiency, often accompanied by activation of facultatively pathogenic microbial

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species. A complex of therapeutic-prophylactic measures aimed at correction of microecological intestinal abnormalities includes: 1) treatment of principal pathology, 2) creating conditions promoting normalization of intestinal microbiocenosis, 3) application of agents with target positive effect on gut microflora.

It was convincingly proved that regular administration of antibiotic leads to pathogen resistance so that formerly efficient medicines lose efficiency [3]. In particular, antibiotic treatment of gastrointestinal diseases results in shift of quantitative and qualitative composition of facultatively pathogenic and normal intestinal microbiota defined as bacteriosis – one of the key factors determining progress of pathologies with diarrhea syndrome.

The afore-mentioned circumstances necessitated development of a novel generation of safe and effective biopreparations aimed at correction of intestinal biocenosis and fostering of animal immunity. In recent years probiotics based on living microbial species and their structural components and metabolites beneficial to gut microbiota find wide use as substitution agents accelerating recovery of natural gastrointestinal microflora in young farm stock after antimicrobial drug therapy. [4, 5]. Unlike antibiotics, they do not affect normal microbiota, are safe and eco-friendly, have no counter-indications.

Search for efficient probiont-bacteria is a priority task of modern biotechnology. Probiotics may be derived either from normal microflora or from saprophytes atypical for gut microbiota capable to replace pathogenic microorganisms from the intestine. Sporulating bacteria of genus *Bacillus* hold a special place among probionts [6]. Probiotic bacillus strains are non-adhesive transitory gut representatives. Valuable properties of these microorganisms make them a vital stock for upgrading biopreparations. Primarily it's enhanced enzyme activity allowing to regulate and stimulate digestion processes capable to the ability to exert antiallergic, antibiotic action and to raise non-specific resistance of microorganism [7, 8]. High antagonistic activity towards a broad spectrum of strictly and facultatively pathogenic microorganisms and auto-elimination from gastrointestinal tract makes production of prophylactic-therapeutic agents from probiotic bacilli especially attractive.

Taking above-mentioned aspects into account, this study was focused on elaboration of novel probiotic product based on efficient strain of sporulating *Bacillus* bacteria for prevention and treatment of enteritis and respiratory diseases in cattle.

## MATERIALS AND METHODS

Bacterial strain *Bacillus subtilis* 9/9 distinguished by elevated antagonistic activity against animal pathogens was chosen for further investigations (deposited at Collection of non-pathogenic microbial cultures, Institute of Microbiology, National Academy of Sciences, Belarus under registration number B 454 D) [9].

Strictly and facultatively pathogenic bacteria from collection of Institute of Experimental Veterinary Research responsible for intestinal and pulmonary infections of farm stock – *Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Salmonella holeraesuis, Staphylococcus sp., Pasterella multacida, Streptococcus sp.* served as test objects to evaluate antimicrobial activity of examined culture.

Submerged fermentation of antagonistic strain was carried out at temperature 30ºC on the nutrient medium with molasses as the carbon source in Erlenmeyer flasks on the shaker (180-200 rpm), in lab fermenters ANKUM-2M of 70 l capacity, industrial bioreactors of 30 l and 100 l volume at aeration rate 1.0 lair/l medium min and agitation rate 200 rpm.

In experiments on optimization of *B. subtilis* B 454D fermentation conditions in lab fermenter ANKUM-2M growth temperature and aeration rates were varied in the following range -30-34ºC and 0.8-1.2 l air/l·min at agitation rate 200 rpm. For each operational regimen growth parameters were calculated and dynamics of antagonistic activity was monitored.

Microbiological and biochemical control of culture development and accumulation of antimicrobial metabolites was performed by taking and analyzing samples of cultural broth, and sporulation process was observed by microscopy.

Finite dilutions technique was applied to estimate bacterial cell titer [10]. Spore concentration was evaluated by the same method upon heating of bacterial suspension at 80ºC during 70 min.

Antagonistic activity of bacteria was assessed by wells [11] and replica methods [12].

Interaction of bacterial antagonists with normal gut flora species was investigated by wells technique, using bifido-and lactobacteria as test cultures [11].

Adhesive properties of bacilli were analyzed according to V.Brilis and H.Lentzner [13] where ram and human erythrocytes stabilized with glutaric aldehyde served as a model.

Toxicological Bacinil trials were conducted at the department of cattle diseases and lethal infections, at vivarium of Institute of Experimental Veterinary Research.

Qualitative and quantitative make-up of gut microbiota in healthy laboratory animals was established by inoculating small intestine contents of mice aged 1 month and weighing 20-22 g onto selective nutrient media. Studies on Bacinil efficiency were carried out at department of viral infections, Institute of Experimental Veterinary Research and at Belarusian farms. The following parameters were controlled: duration of pathology, number of recovered animals, therapeutic efficiency, body weight gains in test and control cattle. Large-scale tests to evaluate Bacinil efficiency for prevention and treatment of gastrointestinal and respiratory diseases were conducted at bull breeding complex Vishnyovka 2002, Minsk region.

The obtained results were analyzed using Microsoft Excel software. Statistical processing of experimental data implied determination of arithmetical means and their confidence ranges for probability level 95%.

## RESULTS AND DISCUSSION

In accordance with obtained findings (Table 1) strain *B. subtilis* B-454 D is able to control spreading of broad spectrum of animal pathogens. Antibacterial action of the culture is expressed as growth inhibition zones of pathogenic bacteria ranging from 20.0 to 31.5 mm in diameter.

It was established by comparative analysis of B. subtilis B454 D antagonistic activity with antagonists of genus *Bacillus*, namely *B. subtilis* BKM 4759 D-active principle of Subalin preparation that the studied culture is not infection to foreign analogs in spectrum of antagonistic activity, and with respect to certain test cultures (*Pasterella multacda, Proteus vulgaris*) exceeds them [14] evidencing good application potential of selected strain for

biological control of animal pathogens. Toxicological tests performed by team from Institute of Experimental Veterinary Reseach have shown that the selected strain is not toxigenic and not pathogenic and hence it may be used in microbiological industry. It was demonstrated by wells technique that strain *B. subtilis* B-454 D did not affect growth of species-constituents of normal gut microbiota in young cattle – *Lactobacillus gasseri*, *Lactobacillus acidophilus*, as evidenced by lack of growth inhibition zone in studied cultures.

Evaluating adhesive properties of *B. subtilis* B 454 D it was found that adhesion index of the selected strain was less than 1, indicating its inability to colonize mucous membrane of gastrointestinal tract and supporting its affiliation to exogenous transitory (auto-eliminating) microflora.

Investigations into impact of metabolites generated by selected bacterial strain on immune system of lab test animals provided results confirming activation of immune humoral factors. It was detected that in 5 days after per oral administration the levels of lysozyme and interferon in blood serum raised by 20-40%.

**Table1. Antimicrobial activity spectrum of strain *B. subtilis* B-454 D**

| Test culture | *B. subtilis* B-454 D | |
|---|---|---|
| | wells technique[I] | replica method[II] |
| *Escherichia coli* 018 | 21.5±0.5 | 95.1 |
| *Escherichia coli* 099 | 23.5±0.4 | 93.5 |
| *Proteus vulgaris* | 25.0±0.6 | 83.0 |
| *Salmonella holeraesuis* | 20.0±0.5 | 74.0 |
| *Klebsiella pneumoniae* | 21.5±0.2 | 71.0 |
| *Pasteurella multocida* | 22.0±0.6 | 85.0 |
| *Staphylococcus sp*. | 30.5±0.3 | 98.3 |
| *Staphylococcus aureus* 6538-p | 28.5±0.4 | 95.5 |
| *Streptococcus sp*. | 31.5±0.6 | 92.5 |

Note – antagonistic activity was assessed: I – via diameter of growth inhibition zone in test cultures, mm; II – as size ration of experimental and control colonies of test cultures, %.

It was shown that optimal conditions for growth and synthesis of antimicrobial metabolites by *B. subtilis* B-454 D in lab fermenter were reached at pH 7.0, temperature 30 ºC. aeration rate 1 l/l·min and agitation rate 200 rpm on modified Meynell medium with molasses as a carbon source [9].

The obtained findings were used for running pilot plant technology of manufacturing new probiotic product for prevention and treatment of enteritis and respiratory diseases of farm stock at facilities of Biotechnological Center, Institute of Microbiology, National Academy of Sciences, Belarus.

Optimization of technological parameters achieved during scale-up of *B. subtilis* B-454 D fermentation process in pilot-plant bioreactors corroborated several correlations revealed in the course of bacterial growth in 10 l laboratory fermenters ANCUM 2M.

It was found that low rate of air supply 0,8 l/l medium·min negatively affected growth characteristics and antagonistic activity of strain-producer. Rise in aeration intensity to 1 and 1.5l/l·min resulted in more complete consumption of nutrient substrate and, as a consequence, increased number of cells and spores in cultural medium. This is accompanied by maximal

antagonistic activity of the strain towards test cultures. Since growth characteristics and antagonistic activity of bacteria *B.subtilis* BIM B454D have only minor distinctions at aeration rates 1 and 1.5 l/l·min and more intense air supply (1.5 l/l·min) triggers enhansed foam generation, aeration regimen 1 l/l·min was accepted as optimal for growth and synthesis of antimicrobial metabolites by *B. subtilis* BIM B454 D. At this aeration rate mass exchange favoring culture development was achieved at rotation of the stirrer 200-220 rpm.

Temperature regimen of culturing bacteria *B. subtilis* BIM B454D (optimum 30ºC) elaborated in lab fermenters ANKUM 2M was not changed in scale-up experiments.

Active consumption of reducing substances (RS) (80% of total amount) and growth of the culture (maximal CFU titer - $3 \cdot 10^9$ by 24 h) was recorded during the first day of fermentation at optimized conditions (aeration rate 1 l air/l l medium·min, temperature 30ºC. agitation rate 200±20 rpm) (Table 2).

Spore titer reached peak value ($1 \cdot 10^9$) by 36-42 h of bacterial growth, whereas maximum antagonistic activity towards various test cultures was registered by 24-48 h (table 2). At longer fermentation period reduction of activity and decrease of cell and spore titer occurred so that continuation of fermentation over 2 days appeared not expedient.

**Table 2. Growth parameters and antagonistic activity of bacteria *B. subtilis* B-454 D cultured in large-scale fermenter**

| Fermentation time, h | pH | Reducing substances, g/l | | Titer | | *E. coli* 099 growth inhibition zone, mm |
|---|---|---|---|---|---|---|
| | | free | inverted | CFU/ml | Spores/ml | |
| 0 | 7.05 | 7.0 | 10.6 | $2.3 \cdot 10^7$ | - | - |
| 6 | 7.05 | 6.75 | 8.55 | $1.0 \cdot 10^8$ | $2.3 \cdot 10^5$ | - |
| 12 | 6.90 | 4.5 | 6.0 | $1.4 \cdot 10^8$ | $1.2 \cdot 10^6$ | 15.0±0.4 |
| 18 | 6.95 | 0.62 | 3.2 | $1.5 \cdot 10^9$ | $1.3 \cdot 10^7$ | 18.0±0.7 |
| 24 | 6.45 | 0.56 | 2.1 | $3.0 \cdot 10^9$ | $1.0 \cdot 10^9$ | 19.0±0.5 |
| 30 | 6.90 | 0.74 | 0.80 | $3.0 \cdot 10^8$ | $1.1 \cdot 10^9$ | 23.0±0.2 |
| 36 | 7.60 | 0.54 | 0.82 | $2.9 \cdot 10^9$ | $8.9 \cdot 10^8$ | 25.5±0.4 |
| 42 | 7.60 | 0.46 | 0.52 | $2.8 \cdot 10^9$ | $1.0 \cdot 10^9$ | 26.5±0.3 |
| 48 | 7.60 | 0.48 | 0.50 | $2.8 \cdot 10^9$ | $8.4 \cdot 10^8$ | 25.5±0.5 |
| 54 | 7.60 | 0.47 | 0.51 | $1.5 \cdot 10^9$ | $8.0 \cdot 10^8$ | 22.8±0.2 |

Probiotic Bacinil produced according to optimized pilot plant technology is distinguished by elevated titers of CFU, spores and antagonistic activity exceeding activity of liquid preparation grown under laboratory conditions by 8-14%. Pilot plant process regulations for manufacturing probiotic product were elaborated based on obtained results.

To optimize probiotic application technology doses and regime of Bacinil administration were tested on young farm stock – calves and piglets.

For enteritis cases 5 groups of calves with malfunctions of gastrointestinal tract were formed, each including 5-8 animals aged 20 days. It was found that a single application of probiotic at 10 ml dose reduces rehabilitations period, yet recurrence of pathology is not ruled out. 15-20 ml dose of probiotic shortens duration of disease by 45.9-49.4 %, results in 100% recovery of treated calves and secures infection will not come back.

Studies on optimization of Bacinil application schedule have shown that the best strategy envisages intake of 15-20 ml doses twice a day for 3 sequential days. The above-mentioned dosage and application procedure enable to rehabilitate 100% of sick calves within 2 days.

To optimize therapy of enteritis cases in piglets, 5 test groups with gastrointestinal problems were formed, each comprising 25-30 heads aged up to 25 days. The experiments demonstrated that optimal single Bacinil dose is 1.5-2.0 ml. It cuts recovery of piglets by 43-45% and decreases mortality rate by 86%.

Administration of probiotic at optimal doses once per diem during 3 consecutive days curtails rehabilitation period by 47-60% in piglets, while Bacinil application twice a day for 72 hours ensures 100% elimination of pathological symptoms.

Based on completed investigations provisional regulations for application of Bacinil to prevent and treat diseases of young farm stock – calves and piglets were compiled.

Large-scale trials of Bacinil efficiency were conducted at Belarusian farms. Pilot-plant Bacinil batch in amount of 1000 doses was produced for further farm tests guided by provisional application regulations.

To study therapeutic efficiency of probiotic Bacinil upon young cattle treatment at Vishnyovka bull-rearing complex in Minsk region, 2 groups of calves aged 40-60 days were formed. Experimental group was subjected to Bacinil treatment at dose 15 ml/per head until complete rehabilitation. Additionally conventional therapy schemes were used. In contrast, Bacinil was not administered to control group. Rehabilitation was assumed to be completed when symptoms of respiratory diseases disappeared. Clinical monitoring of animals was carried out during 14 days. Therapeutic efficiency of probiotic was assessed via duration of pathological process and mortality rate of young stock. Experimental results are presented in table 3.

To evaluate prophylactic efficiency of Bacinil in preventing respiratory diseases of young cattle 2 groups of calves aged 14-20 days were made up. Test group was fed Bacinil at dose 10 ml/head during 7 days. Probiotic was not supplied to the control group. Clinical survey of farm animals occurred for 14 days, including thermometry, general control, feed intake. Prophylactic efficiency of Bacinil was evaluated via incidence of pathologies in young stock. Experimental data are illustrated in table 4.

**Table 3. Therapeutic efficiency of Bacinil upon treatment of respiratory diseases in young cattle**

| Animal groups | Number of animals | Duration of pathology, days | Recovery rate, % | | Mortality rate | | Therapeutic efficiency, % |
|---|---|---|---|---|---|---|---|
| | | | heads | % | heads | % | |
| Test | 60 | 3.5 | 58 | 96.6 | 2 | 3.4 | 96.6 |
| Control | 40 | 8.3 | 25 | 64.5 | 15 | 35.5 | - |

**Table 4. Preventive efficiency of Bacinil with respect to respiratory diseases of young cattle**

| Animal groups | Number of animals | Disease cases | | Mortality rate | | Prephylactic efficiency |
|---|---|---|---|---|---|---|
| | | heads | % | heads | % | |
| Experimental | 20 | 1 | 5.0 | - | - | 95.0 |
| Control | 15 | 3 | 15.0 | - | - | - |

To estimate therapeutic Bacinil efficiency in cases of young cattle enteritis 2 groups of calves aged 5 -20 days were formed. Calves of test group were fed Bacinil at dose 15 ml/animal enterally until recovery. Additionally, they received conventional treatment. Bacinil was not supplied to the control group. Rehabilitation symptoms were defined as disappearance of disbalance in gastrointestinal tract. All animals were subjected to clinical monitoring during 14 days, including: thermometry, general control, feed digestion. Therapeutic efficiency of probiotic was evaluated via duration of pathology and mortality rate of young farm stock. Experimental results are provided in table 5.

**Table 5. Bacinil efficiency in treatment of enteritis cases in young cattle**

| Animal groups | Number of animals | Duration of pathology, days | Rehabilitation rate | | Mortality rate | | Therapeutic efficiency |
|---|---|---|---|---|---|---|---|
| | | | heads | % | heads | % | |
| Experimental | 25 | 3.5 | 24 | 96.0 | 1 | 4 | 96.0 |
| Control | 15 | 6.6 | 10 | 66.7 | 5 | 33.3 | - |

2 groups of calves aged 5-20 days were sorted out to assess preventive efficiency of Bacinil for enteritis cases in young cattle. Calves in test group were fed Bacinil at dose 10 ml/head enterally during 7 days. Probiotic was not administered to control group. Clinical monitoring was carried out for 14 days, including: thermometry, general control, feed digestibility.

Prophylactic efficiency of Bacinil was estimated via incidence of enteritis cases in young farm stock. Experimental results are summarized in table 6.

Summing up, the obtained findings demonstrate high prophylactic (93.3-95.0%) and therapeutic efficiency (96.0%) of Bacinil in cases of enteritis and respiratory diseases of young cattle. The developed product in key parameters – spectrum of antimicrobial action, biological efficiency is unique at Belarusian market and matches best foreign analogs.

**Table 6. Prophylactic efficiency of Bacinil with respect to enteritis of young cattle**

| Animals groups | Number of animals | Recovery rate, % | | Mortality rate | | Therapeutic efficiency, % |
|---|---|---|---|---|---|---|
| | | heads | % | heads | % | |
| Test | 30 | 2 | 6.7 | 0 | 0 | 93.3 |
| Control | 20 | 10 | 75 | 5 | 40 | - |

Following decision of Vetbiopharm Council of Belarus republic Bacinil was registered by Veterinary Department of National Ministry of Food and Agriculture and entered into the list of veterinary products authorized for use in Belarus.

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*Chapter 13*

# PREBIOTICS EXTRACTION FROM *VALERIANA OFFICINALIS L.* EXTRACTION CAKE

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## ABSTRACT

In the work presented the method for the prebiotics obtaining from common valerian extraction cake which remains after obtaining medicinal tincture.

**Keywords:** Extraction, prebiotics, Valeriana officinalis L.

Modern tendencies of healthy nutrition include widening of the choice of foodstuffs balanced in composition and capable of solving a number of preventive tasks. Prebiotics are the most prospective components of functional foodstuffs [1, 2]. Prebiotics provide favourable conditions for the multiplication and metabolic activity of lacto- and bifidus bacteria in the large intestine. Besides they can excrete toxins and improve liver functioning [3,4].

It is now well established that the colonic microflora has a profound influence on health. Consequently, there is currently a great deal of interest in the use of prebiotic as functional food ingredients to manipulate the composition of colonic microflora in order to improve health. Functionally, the human colon undertakes a number of important physiological activities. However, another extremely significant metabolic trait is mediated by gut bacteria. The human embryo is virtually sterile, but at birth microbial colonisation of the gastrointestinal tract occurs, with the neonate receiving an inoculum from the birth canal. During the acquisition period, some bacteria transiently colonise the gut whilst others survive and grow to form the indigenous microflora. Microorganisms occur along the whole length of

the human alimentary tract with population numbers and species distribution characteristic of particular regions of the gut. This allows the establishment of a complex and relatively stable bacterial community in the large intestine. The near neutral pH and the relatively low absorptive state of the colon further encourage extensive microbial colonisation and growth.

Through the microflora, the colon is capable of exhibiting complex hydrolytic digestive functions. This involves the breakdown of dietary components, principally complex carbohydrates, but also some proteins, that are not hydrolysed nor absorbed in the upper digestive tract. The colonic microflora also derive substrates for growth from the diet (e.g. nondigestible oligosaccharides, dietary fibre, and undigested protein reaching the colon) and from endogenous sources such as mucin, the main glycoprotein constituent of the mucus which lines the walls of the gastrointestinal tract. Thus, any foodstuff that reaches the colon, e.g. nondigestible carbohydrates, some peptides and proteins, as well as certain lipids, is a candidate prebiotic according to the above prebiotics definition.

Nowadays, many prebiotics are already used in a broad range of food applications. However, it is still possible to identify desirable targets for enhancement of their efficacy as prebiotics. According to the claims of the producers, these products are effective in supporting the health of human and are also safe.

Prebiotics show both important technological characteristics and interesting nutritional properties. Several are found in vegetables and fruits and can be industrially processed from renewable materials. In food formulations, they can significantly improve organoleptic characteristics, upgrading both taste and mouthfeel. For prebiotics to serve as functional food ingredients, they must be chemically stable to food processing treatments, such as heat, low pH, and Maillard reaction conditions. That is, a prebiotic would no longer provide selective stimulation of beneficial microorganisms if the prebiotic was degraded to its component mono- and disaccharides or chemically altered so that it was unavailable for bacterial metabolism [5].

Most prebiotics are poly- and oligosaccharides [3, 4]. They are obtained either by extraction from plants (e.g., chicory inulin), possibly followed by an enzymatic hydrolysis (e.g., oligofructose from inulin) or by synthesis (by trans-glycosylation reactions) from mono- or disaccharides such as sucrose (fructooligosaccharides) or lactose (trans-galactosylated oligosaccharides or galactooligosaccharides).

Prebiotics can be used for either their nutritional advantages or technological properties, but they are often applied to offer a double benefit: an improved organoleptic quality and a better-balanced nutritional composition. The use of inulin and nondigestible oligosaccharides as fiber ingredients is straightforward and often leads to improved taste and texture. These specific forms of dietary fibre are readily fermentable by specific colonic bacteria, such as bifidobacteria and lactobacilli species, increasing their cell population with the concomitant production of short-chain fatty acids.

These acids, especially butyrate, acetate, and propionate, provide metabolic energy for the host and acidification of the bowel. Lactose occurs exclusively in the milk of mammals and one might speculate from a teleological point of view that lactose ingestion will result in specific benefits for the suckling animal, beyond just being a source of energy. Nowadays, more and more prebiotics are used in functional foods as ingredients which stimulate the growth of health-promoting gut bacteria especially probiotics and offer additional health benefits [5].

One of the accessible sources of glycans is medicinal raw material extract cake. In the paper presented the method for the prebiotics obtaining from common valerian extraction cake which remains after obtaining medicinal tincture was developed.

In Russia *Valeriana officinalis L.* was gathered first at an industrial scale during the reign of Peter the Great. Beginning from XVIII-th century valerian was included into all European pharmacopeiae and in XIX-XX-th centuries it was studied in numerous researches, but it still attracts the scientists' attention. The roots of the plant which are gathered in September and October after the seeds crop are used for medicinal purpose. At this time they contain the most quantity of active ingredients.

Valerian rootstock and roots contain oil, its content varies from 0,5 to 2% depending on botanical form, growing condition and cultivation. The valerian oil contains sesquiterpenes organic acids (valeric acid having spasmolytic effect and formylic, palmitinic, stearinic, acetic, butanoic, apple acid), alkaloids (valerine and chatinine). The rootstock and roots contain fats, resins, magnesium and calcium salts, tanning substances, free amines and carbohydrates [6, 7].

In this work the raw material remaining after the obtaining of medicinal tincture was used for the extraction of valerian polysaccharides. It was preliminary dried in the air at a room temperature for 24 hours. Warm distilled water ($60^0$C) was added to the plant raw material at a ratio 1:10 (weight), the flask was put into the thermostat ($65^0$C) for 3 hours. The extract obtained was filtrated through the gauze, and polysaccharides were isolated by 96% ethanol precipitation from the extract at a ratio 1:3 with the lyophilization followed.

The temperature of extraction ($65\pm1^0$C) is the most optimal as a higher temperature leads to thermal destraction of polysaccharides and a temperature lower than the optimal range does not provide full extraction of polysaccharides from the raw material. It was proved experimentally that the optimal time of extraction of valerian polysaccharides is $3 \pm 0{,}1$ hours that provides maximum degree of polysaccharides extraction. The reduction of the process duration does not allow obtaining the maximum yield of common valerian polysaccharides, while the increase in the process duration is unreasonable as after $3 \pm 0{,}1$ hours the yield of polysaccharides does not increase. The use of water as an extracting agent can be explained by its food and pharmaceutical application; besides the use of other organic solvents (acetone, hexane, ethyl ether) causes denaturation of the polysaccharide extracted. It is impossible to use ethyl alcohol as an extracting agent because the polysaccharides from the extraction cake do not go into alcohol solution.

To determine monosaccharide composition of the polysaccharides obtained the acid hydrolysis was carried out. Into the test tube 0,02% solution of common valerian polysaccharides(15 ml) was placed, chlorohydric acid was added to get 2% solution of HCl, then the tube was put into the thermostat. The reaction was carried out for 2 hours at a temperature of $80^0$C.

The hydrolysates obtained were analysed by the method of highly effective liquid chromatography. During the analysis chromatographic system «Dionex, Ultimate 3000 USA» equipped with comparison refractometer and liquid chromatomassspectrometer was used. The system is also equipped with a peristaltic pump with automatic washing of working plungers, the system of the solvent cleaning, a needle port, analytical column of stainless steel (500x2 mm) with precolumn (50x2 mm). The polymeric carrier Reprogel-H which is a weak cation-exchanger was used as a stationary phase. The column was characterized by 164000

theoretical plates, and the coefficients of peaks asymmetry did not exceed 1,005. The sulphuric acid solution ($C_{H2SO4}=10^{-9}$ mol/l) was used as a mobile phase. The rate of the eluent feed was 0,5 ml/min, the pressure at the column entrance was 24 atm. The chromatographic analysis was carried out at a temperature of 30,5°C. The chromatograms were processed by the method of corrected normalization of the peaks squares.

Monosaccharide composition of common valerian glycans is presented in Table 1.

**Table 1. Polysaccharides composition of common valerian**

| Sugars content (% mass) | | | | |
|---|---|---|---|---|
| ramnose | arabinose | xylose | mannose | galactose |
| 21,2 | 13,2 | 5,2 | 16,2 | 14,8 |

Glucose and D-galacturonic acid were detected.

The polysaccharides obtained were tabletted with KBr to analyse their structure by the method of infra-red spectroscopy. To produce a tablet 1 mm thick and 3mm in diameter 18,4 mg potassium bromide was used. The powder was mixed with substance under study in an agate mortar. The test quantity of the substance studied was chosen within the range of 0,2-1 % of the immersion substance.

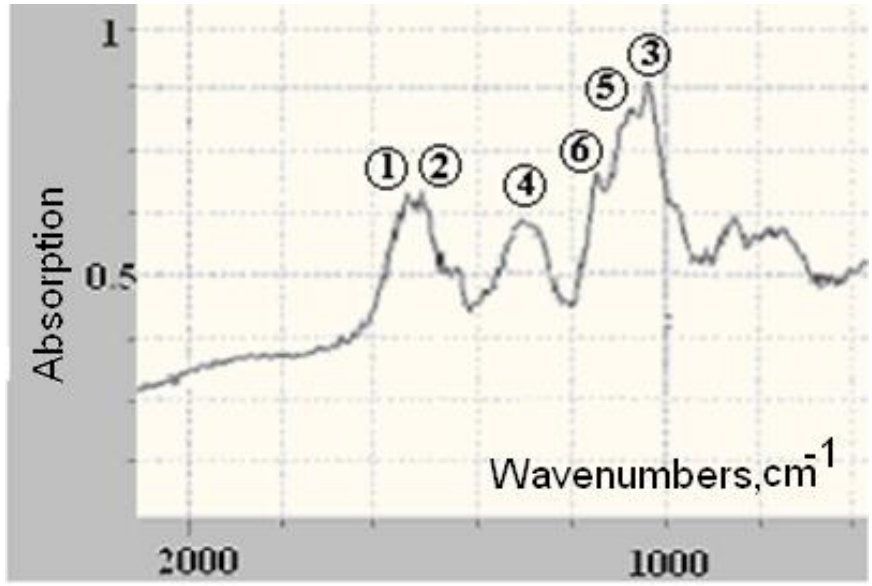


Figure 1. Infra-red spectrum of common valerian polysaccharides.

In the spectrum obtained (Figure 1) there are peaks corresponding to the functioning groups oscillations characteristic of acid polysaccharides: COOH $_{(s)}$ (1540-1580 $sm^{-1}$) (peak 1), COOH $_{(as)}$ (1520-1525 $sm^{-1}$) (peak 2), $\overline{=}$C - O - C$\overline{=}$ (1040-1050 $см^{-1}$) (peak3), (1390-1410 $см^{-1}$) (peak 4), C-C (1150 $sm^{1}$ (peak 6)). A wide peak corresponding to 1078 $sm^{-1}$ (peak 5) allows attributing the polysaccharides obtained to arabinogalactans [8]. The results of the study [9] of the influence of *Valeriana officinalis L.* carbohydrates on the intestine

microflora showed their high effectivity as prebiotics. The use of carbohydrates *Valeriana oficinalis L.* in the complex treatment causes considerable improvement of all intestine microflora characteristics and can be used in the correction of dysbacteriosis.

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*Chapter 14*

# VARIATIONS IN PHYSICAL-CHEMICAL PROPERTIES OF BLOCK-COPOLYMER CHITOSAN WITH METHYL ACRYLATE DURING BIODEGRADATION UNDER THE ACTION OF *ASPERGILLUS TERREUS*

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## ABSTRACT

Variations in physical-chemical properties of the block-copolymer of crab chitosan with methyl acrylate under the action of micromycetes (*Aspergillus terreus*) are investigated. The obtained data have been compared with the characteristics of chitosan and polymethyl acrylate as well as the mixture extracted from the solution of corresponding homopolymers. The standard enthalpy of combustion and chitosan formation have been determined.

**Keywords:** Block-copolymer, crab chitosan, methyl acrylate, biodegradation, Aspergillus terreus

## 1. INTRODUCTION

Biostability and biodegradation of different polymer materials is a very important ecological and engineering problem [1]. There are several aspects of this problem. One of them is elimination of domestic and industrial wastes [2]. It is known that an important role is the processes of material destruction belongs to microorganisms, namely microscopic fungi.

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The activity of enzyme systems, their versatility and lability allow this group of living organisms to use different polymers of both natural and synthetic origin as a food source [3].

On the other hand, ecological significance of biostability is dual: first, protection of materials from the negative action of microorganisms (resource saving) and, second, improvement of the quality of the human habitat since many fungi. Being active biodegradants, are opportunistic pathogenic organisms capable of causing serious diseases of a man [4]. That is why special attention has been paid lately to fabrication of polymer compositions based on natural (chitin, chitosan, starch, and cellulose) and synthetic polymers. The advantage of these materials is associated with their regular stability to the action of microorganisms that allows producing both biostable and, on the contrary, biodegradable compositions.

For the properties of such hybrid polymer compositions to be purposefully controlled, it is necessary to study the effect of the components on their physical-chemical properties. In this connection, we have studied variations in the physical-chemical properties of the block-copolymer of crab chitosan (CHS) with methyl actlate (MA) under the action of micromycetes (*Aspergillus terreus BKM F-1025, Thom, 1918*).

The obtained data were compared with the characteristics of CHS, polymethyl acrylate (PMA), as well as the mixture extracted from the solution of the corresponding homopolymers. The physical-chemical properties of the above compositions were studied using the method of differential thermal analysis (DTA) in the range of 190-350$^{o}$C.

## 2. MATERIALS AND METHODS

The investigations were carried out using chitosan (β-D-1,4-N-glucosamine, $(C_6H_{11}0_4N)_n$) extracted from crab shells manufactured by "Shanghai AZ import & export Co., Ltd" (China). Its molecular mass was $1\bullet10^5$, the degree of deacetylation (DD) was 78%, the molar mass of the repeat CHS unit was 161 g/mol. DD was determined using potentiometric titration in the HCl solution (0.1 N) with NaOH (0.1 N) serving as a titrant [5], while the viscosity average molar mass was measured using a viscosimetric method at 21$^{o}$C in the mixture of $CH_3COOH$ (0.33 N) and NaCl (0.33 N) with the Ubbelohde viscosimeter. The calculations were performed with the formula $[\eta] = kM^{\alpha}$, where $k = 3.41\ 10^{-3}$, $\alpha = 1.02$ [6].

The diluted solutions of CHS in the acetic acid (AA) of the purum grade, State Standard (GOST 61-75) were used. The concentration of the ground substance was 99.5%, $\rho^{20}$=1.049 g/cm$^3$.

Before using, the methyl acrylate ($CH_2$=CH-C(O)-O-$CH_3$) was dried with $CaH_2$ and distilled in the setup with the complete condensation attachment collecting the fraction with $t_{boil}$=80.2$^{o}$C and $\rho^{25}$=0.950 g/cm$^3$.

The oxidation-reduction system based on hydrogen peroxide (ρ(25% solution)=1.4 g/cm$^3$) and ascorbic acid served as an initiator of solution block-copolymerization of MA with CHS. The concentration of the AA in water was 6 mass%, while the concentration of CHS in it was 3 mass%. Block-copolymerization was performed for 24 h at 18-23$^{o}$C. The films based on the CHS/MA block-copolymer were prepared by pouring of reaction mixtures on a lavsan substrate [8].

For the salt form of chitosan to be converted into a protonation one, the films were treated with a 5% solution of NaOH (for 5 min) followed by rinsing in distilled water up to pH 7.

The CHS/PMA mixture was prepared by precipitation with ethanol from the corresponding solutions of homopolymers and the obtained residue was dried in a vacuum cabinet till constant mass. Biodegradation was performed in the presence of strains of *Aspergillus terreus* which, based on the results of the previous studies, proved to be the most efficient for the given compositions [7].

The investigations lasted 20 weeks. The micromycetes were offered by the All-Russia Collection of Microorganisms (Pushino, Moscow Region). To reveal the effect of biodestruction on physical-chemical properties of polymer compositions, three series of experiments were carried out according to State Standard (GOST 9.049-91, method 1) to estimate the contribution of the direct action of micromycetes and the products of their vital activity [9].

In series No.1, the films were placed under the solid completely agarized Chapek-Dox medium (the medium surface was seeded with the fungi), while in series No.2 the specimens were placed on a two-week-old lawn with fungi. The specimens of polymer compositions not subjected to the action of the fungi served as a reference.

The DTA in the range of 190-350$^{o}$C was chosen as the method for investigating physical-chemical properties of the polymer compositions. The design of the device and the experimental technique are described in [10, 11]. Quartz served as a reference.

The charges of the specimen and the reference were 0.2–0.3 g. The specimen temperature and the temperature difference between the specimen and the reference were measured with a chromel-copel thermocouple with an error of 0.5 degrees. The thermocouple was calibrated using a reference platinum resistance sensor and reference substances within the entire temperature range. The experiment was carried out in the helium atmosphere. The heating rate in the experiments was 5 degrees/min.

A deviation from linearity did not exceed 1%. Three heating and cooling cycles were accomplished for each specimen. After the first heating up to 150$^{o}$C, the adsorbed water was removed from the specimen by vacuumization directly in the thermal chamber of the DTA setup and its concentration was determined by weighing.

During the third heating cycle the specimen was subjected to destruction. The enthalpy of CHS combustion was determined in a modified V-08MA calorimeter with a static calorimetric bomb [12]. The calorimetric system was calibrated using the reference benzoic acid of K-2 grade ($\Delta U_c$= –26460.0 J/g when weighed in the air).

The energy equivalent of the system was W = 14805±3 J/degree with a doubled quadratic deviation from the mean of 0.02%. The CHS was burned in the form of pellets embedded in paraffin at the oxygen pressure of $3 \bullet 10^6$ Pa. Based on the data of chromatographic analysis, the oxygen contained the following impurities, mol%: 0.8 of $N_2$; 0.002 of CO and $CO_2$; and 0.001 of hydrocarbons.

The substance in a quartz crucible was ignited by a condenser discharge to a platinum wire connected with the substance via a cotton thread. The temperature rise was measured with two platinum resistance sensors and the SH1516 digital voltmeter connected to the bridge circuit.

**Table 1. Averaged temperatures of physical transitions in crab chitosan, CHS–MA block-copolymer, and CHS/PMA mixture before and after the action of *Aspergillus terreus***

| Specimen | m, g | H2O, mass% | tvap (H2O) oC | ttr, oC | tg(PMA) oC | tβ, oC | tg1, oC | tg2, oC | tdest1, oC | tdest2, oC | Loss of mass with respect to dry , mass% |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Crab Chitosan initial | 0.2446 | 8.9 | 132 | ---- | ---- | 48 | 73.5 | 120.5 | 302 (exo. max.) | 328 (exo. max.) | 41 |
| CHS–MA block-copolymer | 0.5010 | 4.4 | 130 | ---- | 15 | 31 | 72 | 125 | 300 (exo. max.) | 325 (exo. max.) | 16 |
| CHS–PMA mixture*, (through solution) | 0.3247 | 6.7 | 130.5 | ---- | –5.5<br>10.7<br>14.0 | ---- | 71.5 | 120 | 295 (exo. max.) | 328 (exo. max.) | 26 |
| CHS–MA copolymer treated with Aspergillus terreus on the lawn | 0.3002 | 5.2 | 120.5 | –19.5 | 13.5 | 25.5 | 61 | 102 | 323 (endo. min) | 342 (endo. min) | 47 |
| CHS–MA copolymer treated with Aspergillus terreus under the lawn | 0.2705 | 6.8 | 124 | –18 | 13 | 31 | 55 | 117 | 324 (endo. min) | 335.5 (endo. min) | 42 |

*)The dependence of $t_g$(PMA) on the concentration of adsorbed water in the specimen, mass%: 14.0 (0); 10.7 (1.4); –5.5 (5.3)

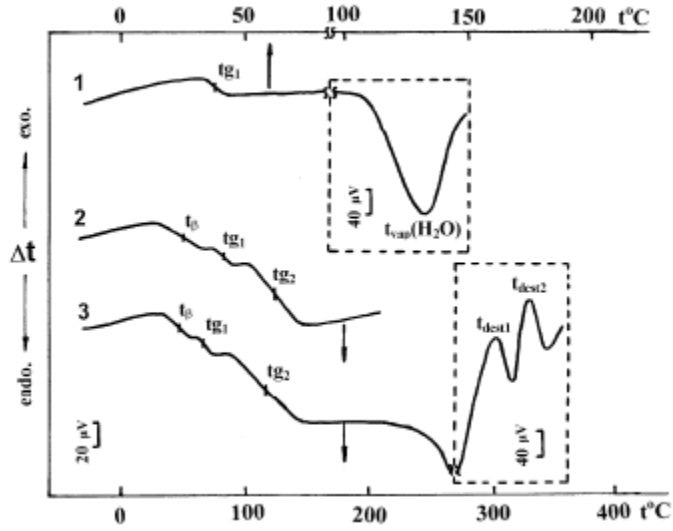


Figure 1. Thermograms of crab chitosan obtained at three repeated heating cycles: (1) the first, (2) the second, (3) the third.

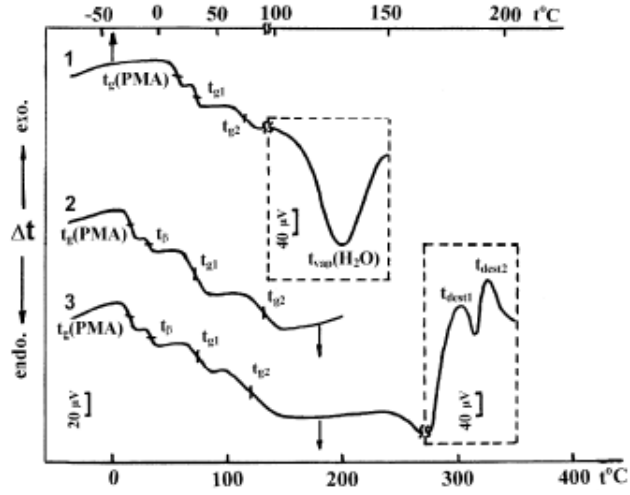


Figure 2. Thermograms of block-copolymer of chitosan with methyl acrylate obtained at three repeated heating cycles: (1) the first, (2) the second, (3) the third.

The mass of the specimen from the analyzed composition was determined based on the amount of $CO_2$ formed during its combustion. The calculations were performed for the following reaction of CHS combustion:

$$C_6H_{11}O_4N\ (s) + 6.75\ O_2(g) \rightarrow 6\ CO_2(g) + 5.5\ H_2O(l) + 0.5\ N_2(g).$$

The energy of combustion of the analyzed substance $\Delta_cU$ was determined under the conditions of a calorimetric bomb. The standard values of $\Delta_cU^o$ and $\Delta_cH^o$ for the CHS combustion reaction were calculated based on the average value of $\Delta_cU$ and the molar mass of the CHS repeat unit, with the Washburn correction and the correction conditioned by

variations in the number of gas moles taken into account. Based on the obtained $\Delta_c H^o$ values, the standard enthalpy of HTS formation ($\Delta_f H^o$) in a solid state at 25°C was calculated using $\Delta_f H^o(H_2O, l) = -285.830 \pm 0.042$ J/mol and $\Delta_f H^o(CO_2, g) = -393.51 \pm 0.13$ J/mol [13].

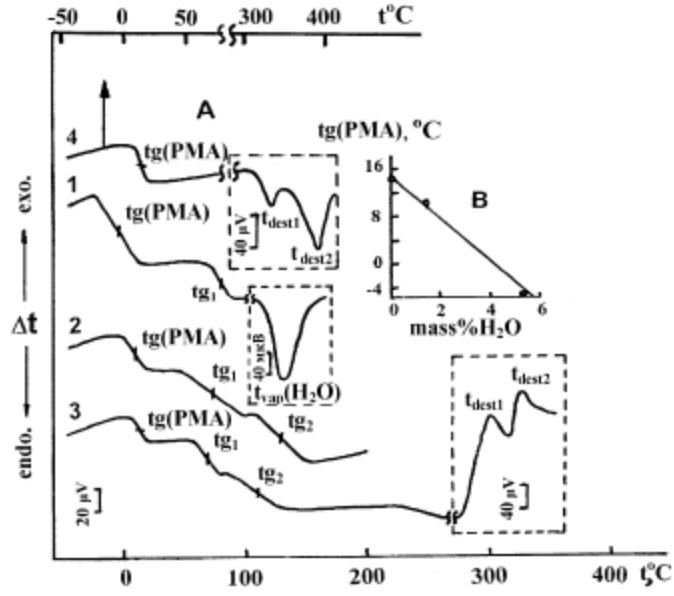


Figure 3. Thermograms of PMA (4) and its mixture with chitosan (3) obtained at three repeated heating cycles: (1) the first, (2) the second, (3) the third. The PMA vitrification temperature in mixtures with chitosan as a function of water concentration in them (B).

## 3. Results and their Discussion

The obtained experimental results are presented in Figure 1–5, while the averaged transition temperatures are listed in Table 1. The performed investigations showed that initial air-dry specimens contained 4.4–8.9 mass% of adsorption water which evaporated from them at 120-132°C (Figures 1–5, curves 1, Table 1).

Several relaxation transitions (a β-transition and two devitrification ones) were observed in dehydrated CHS (Figure 1, curves 2 and 3). It is attributed to a complex molecular and supramolecular structure of the given polysaccharide. The β-transition in CHS is related to oscillations of pyranose rings around a glucoside bond. Chitin and other polysaccharides studied by us behave themselves in a similar manner [10, 14–24]. Irrespective of the source of initial chitin, a distinctive feature of chitosan [21] is two-stage destruction accompanied with the release of energy in the form of heat ($t_{dest1}$ = 302°C, $t_{dest2}$ = 328°C) (Figure 1, curve 3). The loss of mass in this case is 41% (Table 1).

The results of the experiments of determining of the energy of CHS combustion are given in Table 2. The calculated values of $\Delta_c U^o$, $\Delta_c H^o$ and $\Delta_f H^o$ in the solid state at 298.15 K are kJ/mol: −3154.9±13.4 kJ/mol, –3155.6±13.3 kJ/mol and –777.55±13.3 kJ/mol, respectively.

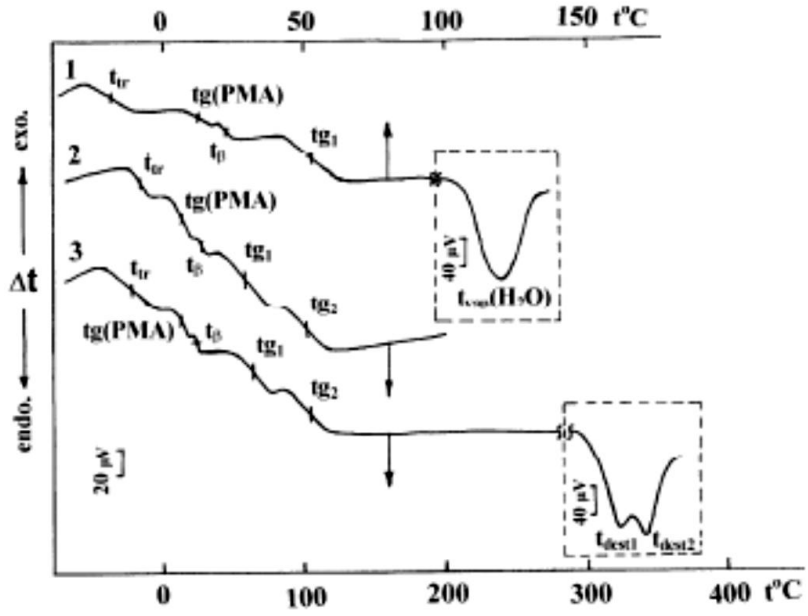


Figure 4. Thermograms of the specimen of block-copolymer of chitosan with MA after the action of *Aspergillus terreus* (series No.2) obtained at three repeated heating cycles: (1) the first, (2) the second, (3) the third.

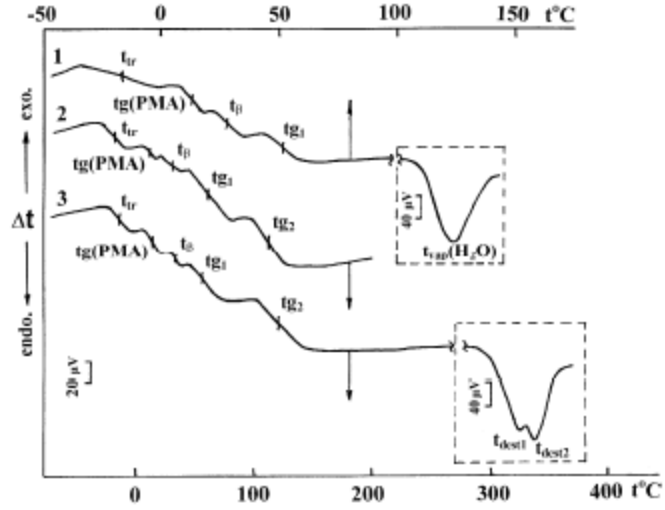


Figure 5. Thermograms of the specimen of block-copolymer of chitosan with MA after the action of *Aspergillus terreus* (series No. 1) obtained at three repeated heating cycles: (1) the first, (2) the second, (3) the third.

Devitrification in the PMA was observed at $t_c$=14.5°C (Figure 3.A, curve 4; Table 1). The value found for PMA in literature [25–27] is $t_c$=6–8°C. However, based on more reliable calorimetric data of B.V. Lebedev [28] $t_c$(PMA) = 12°C. The PMA temperature $t_c$ determined by us was higher as compared to the value obtained when measuring the heat capacity [28] that is likely to be explained by a dynamic mode of specimen heating in the DTA experiments [29].

The PMA destruction is two-staged and it is accompanied by absorption of the energy in the form of heat. The first endothermic maximum is observed at $t_{dest1}$ = 326°C; the second one, at $t_{dest2}$=397°C (Figure 3.A, curve 4). The destruction being completed, the loss of the PMA specimen mass was 87%. Analogous data on the PMA destruction are given by S. Madorsky [30]. When analyzing the products of the PMA pyrolysis it was revealed that a substantial release of volatile products (9.8 mass%) began at 292°C. A sharp increase in the concentration of gaseous products (from 34.8 to 74.1 mass%) was observed at 325–329°C. At 399°C, the concentration of volatile products reached 96.8 mass%.

A CHS/MA block-copolymer is characterized by the transitions typical for both polymers (Figure 2, Table 1). Chitosan does not affect the PMA temperature $t_c$. In its turn, PMA slightly decreases $t_\beta$ of CHS (up to 31°C), does not affect its $t_{c1}$ (72°C), and increases $t_{c2}$ (125°C). The PMA does not practically affect the chitosan destruction (Figure 2, curve 3). Only the loss of the specimen's mass decreases (by 2.5 times as compared to individual chitosan) since the concentration of polysaccharide in the copolymer is 25%.

No β-transition of CHS was revealed in the mixture of homopolymers and its vitrification temperature did not change (Figure 3.A, Table 1). A distinctive feature of the mixture was the plasticizing action of adsorbed water on PMA (Figure 3.B, Table 1). A proportional decrease in $t_c$(PMA) was observed at an increase in the $H_2O$ concentration in the specimen. The destruction of the mixture was similar to that in chitosan (Figure 3.A, curve 3), while the loss of mass in this case takes an intermediate position between CHS and block-copolymer (Table 1).

We revealed variations in the temperature of CHS relaxation transition under the action of *Aspergillus terreus* on the CHS/MA block-copolymer. These variations are observed both under the direct action of micromycetes (series No.2) (Figure 4) and in case of their indirect action taking the effect of the *Aspergillus terreus* vital life products into account (series No.1) (Figure 5). Under the action of the fungi, the specimens of the block-polymer demonstrated a relaxation transition at t<0°C (Figure 4 and 5). It is likely to be related with minor (oligomer) residues of CHS macromolecules formed under the action of micromycetes. The fungi do not affect $t_\beta$ of the chitosan in case of the indirect action of their vital life products, while at the direct action of micromycete flocci $t_\beta$ of chitosan decreases by 5.5°C as compared to the initial copolymer. In both cases, a substantial decrease in its $t_{c1}$ and $t_{c2}$, is observed, the vitrification of amorphous chitosan microregions taking place at the lower temperature if the film is placed under the nutrient medium seeded with *Aspergillus terreus* (series No.1), while highly-ordered microregions vitrify if the film is positions on a two-week-old fungi lawn (series No.2). This case can be related to both partial destruction of highly-ordered CHS microregions and the plastisizing effect on CHS from the side of low-molecular products of the fungi vital life. The temperature of PMA vitrification in this case does not practically change (Table 1).

The effect of the fungi is the most pronounced in the copolymer destruction. The process remains two-staged but, however, it becomes endothermic (Figure 4 and 5, curves 3), i.e., the sign of the energy effect changes. The destruction temperature increases as well ($t_{dest1}$ by 23 and 24°C; $t_{dest2}$ by 17 and 10.5°C). It can hardly be related to the formation of a protein component of the products of the fungi vital life since, e.g., based on our data [31–33] the serum proteins of human blood decompose ay lower temperatures (155–263°C). The obtained temperatures of destruction of the copolymer specimens treated with micromycetes are closer

to the temperatures of PMA destruction (Figure 4 and 5, Table 1). It is not surprising since biodestruction of the blocks of a natural polymer is the first to take place. In this case, the temperatures $t_{dest1}$ almost coincide, while $t_{dest2}$ of the PMA decreases by 55°C (series No.2) and 61.5°C (series No.1). Variations in $t_{dest}$ of the PMA in the block-copolymer can point to partial destruction of its synthetic fragments as well.

m- mass of combusted substance; Δt – temperature rise in the experiment with the correction for heat exchange; $q_{par.}$, $q_{ct}$, $q(HNO_3)$, $q_c$, – corrections for the energy of combustion of paraffin, cotton thread, the energy of formation of $HNO_3$ water solution, and incomplete combustion of carbon, respectively; $\Delta_c U$,-energy of combustion of the studied substance under the conditions of a calorimetric bomb.

**Table 2. The results of experiments on determining the energy of CHS combustion at 25°C**

| m, g | Δt, J | $q_{par}$, J | $q_{ct}$, J | $q(HNO_3)$, J | $q_c$, J | $-\Delta_c U$, J/g |
|---|---|---|---|---|---|---|
| $0.2155_5$ | 2.523305 | 33120.7 | 36.0 | 10.0 | 28.8 | 19577.6 |
| $0.2379_1$ | 2.572280 | 33455.4 | 32.1 | 14.7 | 35.1 | 19401.5 |
| $0.2644_4$ | 2.591835 | 33184.2 | 31.5 | 12.3 | 39.3 | 19602.3 |
| $0.2420_7$ | 2.570570 | 33263.4 | 33.8 | 8.8 | 32.8 | 19764.0 |
| Среднее значение | | | | | | 19586.4±83.7 |

## CONCLUSION

1. The comparison of physical-chemical properties of chitosan, its block-copolymer with methyl acrylate, and the CHS/PMA mixture showed that the polysaccharide does not practically affect the synthetic component, while PMA yields variations in the temperature of the chitosan relaxation transitions. The MA blocks in the copolymer decrease the temperature of the β-transition and increase the temperature of vitrification of the ordered CHS microregions. As far as the mixture is concerned, PMA blocks the oscillations of pyranose rings of CHS and, thus, suppresses the β-transition of the latter.
2. The investigations performed using the DTA method revealed variations in physical-chemical properties of the CHS/MA block-copolymer under the action of Aspergillus terreus. In this case, chitosan is the first to be reprocessed by micromycetes, since it is the temperatures of the chitosan relaxation processes that change most substantially. The effect of Aspergillus terreus also manifests itself in the formation of a new relaxation process at t<0oC. It should be also noted that the effect of micromycetes on the CHS/MA block polymer yields a more substantial change in its physical-chemical properties in case of a direct action of micromycetes (series No.2) since both the flocci of the fungi and the products of their vital life penetrate into the copolymer. An important sign of the micromycete action on chitosan is the disappearance of endothermic maxima of its destruction on the thermograms and the appearance of endothermic maxima of PMA destruction. A decrease in the temperature of the second endothermic maximum of the PMA destruction by 60–

65oC under the action of Aspergillus terreus on the block-copolymer allows assuming that PMA is also subjected to biodestruction.

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*Chapter 15*

# THE DESTRCTION OF THE COMPOSITIONS BASED ON CHITOSAN COPOLYMERS WITH ACRYLIC MONOMERS BY MICROMYCETES

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## ABSTRACT

The article covers the process of the biodestruction of block and grafted copolymers of chitosan with acrylic monomers (methylacrylate, acrylamide, acrylonitrile) under the action of various species of microscopic fungi and their single ferments: oxidoreductases (catalase, peroxidase) and hydrolases (chitosanase, protease, esterase). The most effective destructors for all the studied compositions are the following: Aspergillus niger, A. terreus, Chaetomium globosum, Penicillium cyclopium, P. funiculosum, Trichoderma viride. The example with the usage of chitosan copolymers with methylacrylate exhibits that the destruction process involves synthetic fragments of macrochains which can be concluded from a triple decrease of molecular mass and a drop in physical- mechanical properties of polymer mats. The molecular mass of the fragment of the synthetic polymer in block and grafted (co)polymers is proved to be the limiting factor in the process of the design of totally biodegradable polymers.

**Keywords:** Biodestruction, micromycetees, ferments, block and grafted (co)polymers, acrylic monomers, chitosan, physical- mechanical properties, molecular mass

## INTRODUCTION

One of the acute ecological problems, which drew public attention 20 years ago and remains almost unsolved up to the present moment, is a dramatic increase of the rate of

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industrial waste pollution and the usage of polymer materials. Experts state that by 2010 the production of polymers had amounted 260 mln. tons and the estimated annual growth of their production is going reach 5.5% per year aggravating the current pollution situation. There can be observed a solution of the pollution problem which consists in the implementation of innovative biotechnology. So, two directions can be traced in the development of biotechnology:

- The production of biodestructive polymers on the basis of renewable natural materials (starch, corn);
- The production of biodestructive polymers on the basis of synthetic and natural polysaccharides (starch, cellulose, chitin, chitosan) [1].

The researches aimed at the creation of the biodestructive compositions of this type are described mainly in patent literature [2-8]. The world's leading companies such as BASF, DuPont, BAYER have already started the production.

The main achievements in the creation of the biodegradable materials and the unsolved problems in this field are presented in scientific reviews [9-10]. It is remarkable that all the analysed polymers are heterogeneous-chain polymers containing essential and amide connection, and natural polysaccharide polymers. Yet these achievements do not resolve the contamination problem caused by the production and usage of large and middle polymers on the basis of the monomers of vinyl row, which will definitely be manufactured by Russian and foreign producers in the nearest decades.

Thus, the task of the creation biodegradable materials based on these particular polymers is urgent.

At present there is a limited number of regular fundamental studies revealing the impact of particular factors upon the process of the biodestruction of polymers of various chemical structure and carbon-chain ones in particular [9-10]. To understand the mechanism of biodegradation it is important to consider not only the physical-biological aspects of the process such as temperature, oxygen, moist, microorganism impact, the chemical nature of the polymer itself, but also its biodegradation products, molecular mass (the effect of the molecular mass of the copolymer and the length of sections of the synthetic fragments of its chains is being highly debatable now) which is essentially important [9].

The latter play an important role in the organization of the supramolecular structure of the polymer, the development of crystallinity zones in it, enzyme-absorption active centres, and probably in their activity. A comprehensive approach to the assertion of the dependence of (co)polymer biodestruction ability on their structure, compound and molecular mass with the implementation of modern physical-mechanical, analytical and rheological research methods will give an opportunity to obtain new data about the bioconversion of polymer compositions and assert the fundamental principles of the creation of the materials with biodestruction-time control based on the polymers of vinyl row combined with natural ones.

Thus, the purpose of the given research is to study the mechanisms of the initial biodegradation stages of the compositions based on natural polymers and synthtical vinyl-row ones.

## EXPERIMENTAL CONDITIONS

The study objects are the films based on block- and grafted copolymers of chitosan with vinyl monomers (methylacrylate monomers (methylacrylate, acrylamide, acrylonitrile) and mixtures based on them.

The synthesis of the (co)polymers involved chitosan (CTS) ["Биопрогресс" ("Bioprogress") and "Биокомбинат" (Biocombinat), Moscow DC], produced form crab shells with the molecular mass (MM) $120\times10^3$ and the deacetylation degree 80-82%. The refinement of the vinyl monomers was standard.

The chitosan grafted copolymers with methylacrylate (MA) (sample №1 – Grafted, Grafted CTS - MA), acrylonitril (AN)(sample №5 – Grafted, Grafted CTS - AN) were produced in aqueous-acetous solutions of the polysaccharide 3% and acetic acid 6%. The initiator was 2.2' –azobisisobutyronitrile (0.01 mole per liter) at 333K within 3-4h. The proportion [MA]/[the element of glycosamine]=3 mole/base mole.

Sample №2. The block-copolymers of chitosan with methylacrylate (Block CTS-MA) were produced in aqueous-acetous solutions of the polysaccharide 3% and acetic acid 6%. at 291-296K with redox initiating system ($\nu(C_6H_8O_6)/\nu(H_2O_2)=1$ in mole, $\nu$(the element of glycosamine)/$\nu(H_2O_2)=50$ in base mole/mole added, the proportion variation [MA]/[the element of glycosamine] was ranged from 2.5 to 3.3 mole/base mole).

Sample №3. To synthetise the grafted copolymer of chitosan with acrylamide (Grafted CTS-AA)

1% polysaccharide solution in 0.4% aqueous solution of acetic acid was used. The necessary amount of the chitosan solution was placed in the reactor after which 9.5 mole of AA per a mole of the glycosamine elements was added. The system was agitated until complete resolvation of AA, was blown through with argon during 20 min., the temperature in the reactor was raised up to 328K and after that the initiator $(NH_4)_2S_2O_8$ ($0.5*10^{-3}$ M) resolved in water was added into the reaction mixture. The process lasted for 3h.

Sample №4 is a mixture of the grafted copolymer chitosan-acrylamide with polyacrylamide (PAA) in the mass proportion of 1:1.7.

The films based on block and grafted copolymers of chitosan with vinyl monomers were produced by pouring their solutions on glass substrates. To transform a saline form of chitosan into an unprotonated one the sample films №1,2,5 were processed with 5% NaOH solution (for 5 min) and ater that rinsed with distilled water until pH 7.

The mechanical strength of the films (before and after the effect of the fungi) was analysed with the tensile-testing machine ZWIC Z005 (State Standard 270-64) at the extension rate of 50 mm/min. The tensile strength ($\sigma$) and relative extension ($\varepsilon$) of the films was calculated by the following formulae:

$$\sigma = \frac{P}{S}, \qquad \varepsilon = \frac{l_1 - l_0}{l_0} \times 100,$$

in which $\sigma$ - tensile strength, $N/m^2$; P – imposed load, H; S – cross-section area of the film, $m^2$, ε – relative extension, %; $l_0$ – sample original length, cm; $l_1$ – sample length preceding the break, cm.

The fungi-effect resistance was measured by State Standard 9.049-91 [11], method 1. The given method enables to estimate the natural fungi-effect resistance of the materials, i.e. the ability of micromycetees to use them as a nutrition source. The gist of the method consists in the following: a certain polymeric composition is placed in Petri dishes and inoculated with a fungi-spores suspension ($1\times10^6$ per ml), put into the thermostat to expose at the temperature of 28±2º C and the humidity of ≥ 95 % for 28 days.

The fungi-effect resistance was visually evaluated by marking the fungi-growth rate on the tested polymers with points 1 – 6. The fungi were cultivated on the Capek-Docx carbon-impoverished (1 g of sucrose per 1 l of the medium) liquid nutrient medium on the shakers ASD – 4M (Automatic Shaking Device) at the speed of rotation of 180 promptness orb/min.

On the fourth cultivation day 2.5 g of the corresponding polymeric compositions were added to the tested samples.

The cultivation longevity was 12 days after which the mycelium was filtered, and the activity of the corresponding exoferments in the cultural medium was measured. The specific activity calculation was executed per mg of protein. The control variants were the ones with no polymeric compositions added to the nutrient medium. Each polymeric composition was subject to two series of experiments four times.

The ferment activity was measured with the spectrophotometer (SP-2000): catalase - at $\lambda_{max}$ = 240 nm [12], the substrate was 30 mM hydrogen peroxide, peroxidase – at $\lambda_{max}$ = 535nm [13], ], the substrate was 0.03 % hydrogen peroxide and 0.1 M paraphenylendiamin (by the Aurand method). The presence of the chitosanase activity was measured by the spectrophotometer by their ability to saccharificate chitin/chitosan molecules. The sugar concentration was measured by the reaction with 3,5-dinitrosalicylic acid. The method is based on a red-coloured compound as a result of the reaction between glucose and 3,5-dinitrosalicylic acid [14].

The measuring of protease activity was conducted by the Anson method [15]. The ferment activity was judged by the quantity of the free tyrosine generated after substrate proteolysis, and the tyrosine in short peptides. The esterase activity was measured by the titrimetric method by the degree the ferment hydrolised the substance which was dioctyladipinate stabilisated with a 2% water-solution of polyvinyl alcohol [16].

The measuring of protein was conducted by the Louri method [17].

The destruction rate of the film materials by the fungi was controlled by the change in the physical--mechanical properties (σ,ε) of the films and the PMA chain-length distribution in the (co)polymers. To identify the MM blocks and grafted chains of PMA the destruction of the chitosan unit of copolymers with $NaNO_2$ was conducted [18]. PMA was identified by the method infrared spectroscopy (spectrophotometer Infralum FT-801).

The chain-length distribution (CLD) of the blocks and grafted chains of PMA was measured by the method of gel filtration at 40 $^0$C temperature on the liquid chromatograph Prominence LC-20VP «Shimadzu» with a column set filed with polystyrol-divinylbenzol standards with $10^6$ and $10^5$ Å pore size. A differential refractometer was used as a detector. The eluent was tetrahydrofuran. The calibration was executed in accordance with narrow-disperse standards.

**Table 1. The investigation of the resistance of some polymeric compositions to the micromycetes**

| № | The material | The species of fungi / the intensity of the mould growth, marks | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Method 1 (State Standard 9.049-91) | | | | | | | | | | |
| | | A. oryzae BKM F-2096 | A. niger BKM F-1119 | A. terreus BKM F-1025 | Chaet. globosum BKM F-109 | Paecil. variotii BKM F-378 | Penic. funiculosum BKM F-1115 | Penic. chrysogenum BKM F-245 | Penic. cyclopium F-245 | Trich. viride F-1117 | The association of fungi | The active biodegrators |
| 1 | 135Grafted Chitosan – methylacrylate polymer | 3 | 5 | 3 | 4 | 4 | 4 | 4 | 5 | 2 | 4 | A. nig., P. cyclop. |
| 2 | Block. Chitosan – methylacrylate copolymer | 3 | 3 | 5 | 5 | 3 | 4 | 4 | 5 | 4 | 4 | A. terr., Chaet. globos., P. cyclop. |
| 3 | Grafted 135polymer Chitosan – AA | 2 | 2 | 2 | 3 | 1 | 3 | 1 | 1 | 1 | 2 | P. funicul., A. terr. |
| 4 | The mixture of the grafted copolymer Chitosan-AA+PAA | 1 | 2 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | terr., Chaet. globos. |
| 5 | Grafted Chitosan-AN polymer | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 4 | P. cyclop., Tr. viride |

## RESULTS AND ARGUMENT

As a result the (co)polymers of chitosan with vinyl monomers were obtained, and their biodestruction ability and the one of individual homopolymers under the effect of microscopic fungi were studied. For test-cultures standard fungi cultures for RCM (Russian Collection of Microorganisms) were opted which are active polymer biodegraders by State Standard 9.049-91. Table 1. presents the results of the growth assessment of the standard micromycete cultures on the studied polymer materials. The analysis of the results shows that the given compositons can be biodegraded by micromycetees. But for all that their this ability differs under the effect of different fungi. The grafted chitosan with methylacrylate, block-chitosan copolymer with methylacrylate, grafted chitosan copolymer with acrylonitril turned out to be utilized more easily with the fungi as compared to chitosan (co)polymers with acrylamide as the latter was biodegraded with a smaller species range of the fungi. Aspergillus niger, A. terreus, Chaetomium globosum, Penicillium cyclopium, P. funiculosum, Trichoderma viride were the most active destructors of all the studied compositions.

Table 2 presents the data of the stability of separate polymer-composition components against the effect of micromycetees. A remarkable fact is that individual homopolymers (polymethylacrylate, polyacrylonitril)which are components the biodegradable (co)polymers, showed resitance against the effect of the fungi, meanwhile chitosan and polyacrylamid did not. This fact proves the hypothesis we made before that the fungi resistance of polymer compositions (copolymer or mechanical) can not be judged if only the fungi resistance of their components is considered [19]. At the same time a series of biochemical experiments to assess the role the role of separate ferments of the fungi such as oxidoreductase (catalase, peroxidase) and hydrolase (chitosan, protease, esterase) in the destruction the synthesized polymer compositions. In this series of experiments the cultures of two fungi species Aspergillus terreus and Penicillium cyclopium which are most active biodegraders of the studied polymer compositions. The results of the given experiment series are displayed in tables 3 and 4.

In all the variants of the experiment a considerable increase of chitosanase activity in comparison with the control sample which testifies to the fact that chitosan as a natural component of the polymer compositions is a considerably available nutritional source. The activity increase of chitosanase under the effect of fungi cultures Aspergillus terreus and Penicillium cyclopium upon various polymer compositions was not the same, which can be connected with physical-biochemical peculiarities of the fungi as long as with the chemical components of the polymers (tables 3, 4). Not in all the variants of the test the synthetical part of the compositions was used by the fungi. In a certain way this fact agrees with the phenomenon diauxic growth of the microorganisms, i. e. the substrate which is more available is utilized first [20]. An increase of the activity of alkaline protease Aspergillus terreus with grafted copolymer chitosan: AA is registered, which is caused by the amide link decomposed by the fungi protease. Thus both chitosan and the functional groups of polyacrylamide provide the growth of the fungi. An increase of the activity of exooxydoreductase (catalase, peroxydase) In the process of the destruction of the grafted copolymer of chitosan with acrylonitril is also registered. These ferments are in some way also supposed to take part in the destruction of the given polymer. So, the analysis of this series of the experiments shows that the initial destruction stages mainly affect the natural part of the polymer compositions –

chitosan as in all the test the chitosanase activity increases meanwhile their synthetical part remains intact. Does it mean that the synthetical part is not involved in the destruction process? We can suppose that its destruction will take place later when chitosan is totally destroyed.

**Table 2. The resistance of some polymers to micromycetes (associative culture, State Standard 9.049-91)**

| Polymer | The fungi resistance in marks (method 1) | The estimation of the fungi resistance |
|---|---|---|
| Chitosan | 5 | Not resistant |
| Polymethylacrylate | 1 | Resistant |
| Polyacrylamide | 3 | Not resistant |
| Polyacrylonitril | 2 | Resistant |

**Table 3. Exoferments activity of *Aspergillus terreus* during the process of the destruction of the polymers**

| Sample | Ferments | Activity | |
|---|---|---|---|
| | | Experiment | Control |
| Grafted copolymer chitosan : AA [AA]/[ glycosamine]= 9,5 mole/base-mole | Chitosanase (glycose mg /protein mg ×hour) | 708,00 | 13,40 |
| | Proteinase acidic (мсМ tyrosine/mg protein×hour) | trace | trace |
| | Proteinase alkaline (мсМ of tyrosine/mg of protein×hour) | 432,00 | Trace |
| Grafted copolymer chitosan : AH [AH]/[ glycosamine]= 3 mole/base-mole | Chitosanaза (mg of glycose/mg of protein×hour) | 893,00 | 54,00 |
| | Peroxidase (conventional unit, c.u.) | 25,02 | 10,32 |
| | Catalase (c.u.) | 91,23 | 24,78 |

$p<0,05$.

**Table 4. Exoferments activity of *Penicillium cyclopium* during the process of the destruction of the polymers**

| Sample | Ferments | Activity | |
|---|---|---|---|
| | | Experiment | Control |
| Grafted copolymer chitosan : MA [MA]/[ glycosamine]= 1,9 mole/base-mole | Chitosanaза (mg of glycose/mg of protein×hour) | 957,00 | 152,00 |
| | Lipase (ml of 0,05N NaOH/mg of protein×hour) | 1,20 | 1,98 |
| Block-copolymer chitosan : MA [MA]/[ glycosamine]= 2,5 mole/base-mole | Chitosanaза (mg of glycose/mg protein×hour) | 191,00 | 23,00 |
| | Lipase (ml of 0,05H NaOH/mg protein×hour) | 3,45 | 6,46 |

$p<0,05$.

To prove the hypothesis experiments were conducted to measure the change in the molecular mass and physical-mechanical properties (σ, ε) of the polymer compositions within a long-time exposure to the fungi (their aggressive metabolites). Penicillium cyclopium and Aspergillus terreus were opted as micromycetees-biodegraders which are most active for grafted and block-copolymers of chitosan with methylacrylate. Both the direct effect of exometabolites (when the fungi were cultivated on the polymers) and indirect one (the effect of metabolism products of the fungi) were studied. The experiment longevity was 20 weeks. The physical-mechanical properties of the films based on grafted and block copolymers of chitosan with MA and molecular mass distribution of PMA chains in (co)polymers were measured on the 2$^{nd}$, 4$^{th}$, 6$^{th}$, 12$^{th}$ and 20$^{th}$ exposition weeks. The results are shown in the tables 5, 6 and figure 1 – 2. Tables 5, 6 display that in case with both the grafted and block copolymers there observed a decline of physical-mechanical properties - failing stress and break strain which testifies to the biodegradation of the polymers. In control tests these properties do not change. It is disclosed that the reaction of the compositions to the exposition to various fungi species Penicillium cyclopium, Aspergillus terreus and their metabolism products differs considerably. The declining of the stress-strain properties of the block-copolymers (table 5) under the effect of Aspergillus terreus unvaries within the whole experiment period (20 weeks), and the films almost completely lose their plasticity (the sample was placed on the fungi culture).

**Table 5. The dependence of the change of the physical-mechanical properties of films of hybrid block-copolymer CHITOSAN-MA from the time of fungus A.terreus effect. ([MA]/[ glycosamine]= 3,3 mole/base-mole.)**

| Time, weeks | σ, MPa | | | ε, % | | |
|---|---|---|---|---|---|---|
| | control | The films were placed under the agar medium of the fungi culture | The films were placed on the fungi culture | control | The films were placed under the agar medium of the fungi culture | The films were placed on the fungi culture |
| 0 | 36,0 | 36,0 | 36,0 | 17,0 | 17,0 | 17 |
| 4 | 37,0 | 29,5 | 21,7 | 20,0 | 6,8 | 3,6 |
| 6 | 33,6 | 28,6 | 18,7 | 18,0 | 5,0 | 3,1 |
| 12 | 36,8 | 28,6 | 11,9 | 17,0 | 2,6 | 3,3 |
| 20 | 35,9 | 28,0 | - | 16,0 | 3,2 | - |

**Table 6. The dependence of the change of the physical-mechanical properties of films of the hybrid grafted copolymer CHITOSAN-MA from the time of fungus P.cyclopium effect ([MA]/[ glycosamine]= 3,3 mole/base-mole)**

| Time, weeks | σ, MPa | | | ε, % | | |
|---|---|---|---|---|---|---|
| | control | The films were placed on the fungi culture | Suspension | control | The films were placed on the fungi culture | Suspension |
| 0 | 32,0 | 32,0 | 32,0 | 18,0 | 18,0 | 18,0 |
| 2 | 29,0 | - | 29,0 | 19,0 | - | 28,0 |
| 4 | 28,9 | 26,4 | 28,9 | 20,0 | 37,9 | 26,0 |
| 6 | 27,0 | 22,1 | 22,0 | 19,0 | 7,6 | 7,0 |
| 20 | 26,0 | 23,3 | 19,0 | 18,1 | 7,3 | 8,0 |

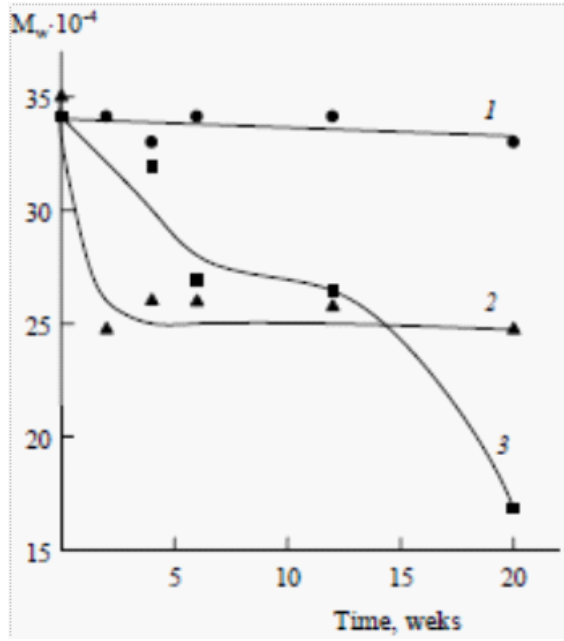


Figure 1. The dependence of the change of the molecular mass of the polymethylacrylate from the time of the fungus A.terreus effect on the hybrid block-copolymer CHITOSAN-MA: 1-control, 2- the films were placed under the agar medium of the fungi culture, 3- the films were placed on the fungi culture ([MA]/[ glycosamine]= 3,3 mole/base-mole).

The effect of the metabolism products of the fungi Aspergillus terreus upon the physical-mechanical properties of the films is inconsiderable (the sample was placed under the agar medium of the fungi culture). The exposition results in case with the grafted copolymer differ. The objective of the given experiment series was to study the physical-mechanical properties of the film samples under the effect of Penicillium cyclopium upon the films placed on fungi culture and under the effect of inoculation of the spore suspension upon the film samples (suspension variant). In the two variants both the strain at failure and failing stress properties decreased 1.5 times. It was noticed that the given decrease was observed within the first six weeks. After that period no changes in the physical-mechanical properties are observed. It turned out that a considerable influence upon the physical mechanical film properties was not only the metabolism of the fungi itself but simultaneous effect of the fungi life activity products (suspension variant).

The change in the physical-mechanical film properties is connected with the destruction the (co)polymer structure. The fungi hyphae penetrate into the polymer film and destroy gradually not only its supramolecular structure but also its macrochain structures.

Figure 3 shows the photographs of the results of the effect of the micromycetes upon the films after 2 and 12 weeks which give an opportunity to visually estimate the bioconversion. It can be clearly seen in the photographs that all the samples placed on the fungi culture are almost completely covered with fungi hyphae after 12 weeks. The samples placed under the agar medium of the fungi culture have even colouring probably due to the chemical interaction of the fungi life activity products with the film components which, as it was said previously, which also tells on their physical-mechanical properties.

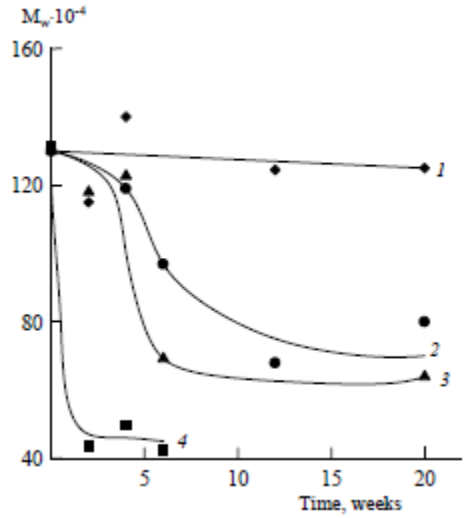


Figure 2. The dependence of the change of the molecular mass of the polymethylacrylate from the time of the fungus P.cyclopium effect on the hybrid grafted-copolymer CHITOSAN-MA: 1-control, 2- the films were placed on the fungi culture, 3- the films were placed under the agar medium of the fungi culture, 4-suspension ([MA]/[ glycosamine]= 3,3 mole/base-mole).


Figure 3. The photographs of the films in 2 and 12 weeks after A.terreus inoculation.

The comparison of the obtained results with the MMD change data (fig. 1, 2) shows that namely within the first six weeks an abrupt decrease of the molecular mass of PMA chains as components of block and grafted copolymers of chitosan-methylacrylate. The molecular mass of the grafted chains decreases three times from 1,3*106 to 4,0*105 and remains almost without change further. In case with the block copolymers there is also a correlation between the change in the physical-mechanical properties and MM. Both the parameters change steadily throughout the whole experiment.

In such a way both in case with the block copolymers and the grafted ones a considerable change in MMD of the synthetical compound of the (co)polymer on the sixth week of the experiment is connected with the consumption of PMA by the fungi.

On the basis of the obtained results the following conclusions can be drawn:

- The synthetical polymers modified by the natural ones are involved in the fungi metabolism and are prior to biodestruction;
- The MM of a synthetical polymer strongly affects the biodegradation of the material. There is an optimal rate of the MM of a synthetic polymer ($4{,}0*10^5$ - $6{,}0*10^5$) which makes possible to create a totally biodegradatable material.

The given results testify to a probability of the creation of hybrid (natural-synthetic) chitosan polymers with methylacrylate with high physical-mechanical qualities and adjustable biodegradation period.

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*Chapter 16*

# BRASSINOSTEROIDS AS POSSIBLE NANOREGULATORS OF BIOLOGICAL SYSTEMS

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## ABSTRACT

Currently the brassinosteroid plant hormones has attracted attention of the scientists, who are engaged in the synthesis and extraction of natural compounds, studies of their biological properties, development of new specimens for agricultural needs, due to their extremely high biological activity. As per their structure brassinosteroids are close to steroid hormones of animals, they have specific influence on the cell membrane permeability, due to the system of adenylate cyclasa they have impact on the activity of definite enzymes (biocatalysts, ferments), regulating the metabolic process and functions of the cells, and directly on the functions of the cells genome. Concentrated in the growth segments of browses and pollen, brassinosteriods penetrate the organisms of invertebrates and vertebrates, causing different specific and general protective effects.

**Keywords:** Brassinosteroids, biological systems, hormone activity, antioxidant processes, nanoregulators, plants, animals

## 1. INTRODUCTION

The role of nanotechnology has significantly grown during the recent decade. Nanobiotechnology has become an independent interdisciplinary field of science. Expected time of wide implementation of modern researches is the mid-21st century. Due to their extremely high biological activity the brassinosteroid plant hormones has currently attracted attention of the scientists, who are engaged in the synthesis and extraction of natural compounds, studies of their biological properties, development of new specimens for

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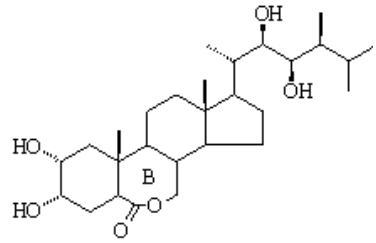
agricultural needs. Plant hormones are low molecular organic substances that are produced by plants and possess regulating functions. Low concentrations (lower than $10^{-11}$ M) of plant hormones are active, with plant hormones causing various physiological and morphological changes in the cells and systems of the organism which are sensitive to their influence.

In 1970 first reports of extracting lipid fraction from the pollen of rapeseed and alder appeared; the extracted fraction possessed marked growth-regulating abilities. Active components of this fraction were called brassinosteroids. Treatment of bean germinants with brassinosteroids proved to increase the seed yielding capacity by 40%. Molecular structure of the active component of new plant hormone group was defined in 1979. Brassinolide proved to be this active component.




Brassinolide and its brassinosteriod analogues differ from other steroids due to lactone B cycle unique to their structure. By now it has been determined that brassinosteroids act as hormones increasing yield of the overwhelming majority of crops: rice, wheat, beans, corn, being effective exclusively in the small (10-40 mg/ga) doses. Brassionosteriods improve plant resistance to ambient adverse conditions: low temperatures, salinization, aridness, cold winds, toxic chemicals etc. Discovered favourable effects of this steroid group are supposed to stem from the ability to adjust the level of corresponding plant hormones in the organism of the plant according to the stress [1].

The structure of brassinolide plant steroid was defined employing spectral methods and X-ray crystallographic analysis. As per the results brassinolide plant steroid belongs to the class of steroids and has a unique lactone B cycle structure, characterized by trans-AB-articulation with 22R lateral chain, 23K diol groups as well as α-cis-diol group in A cycle. The length of molecule is up to 6 nanometers, and its width is up to 1 nanometer. Molecules of this size can easily penetrate any capillary and biological pores.

## 2. BRIEF OF PREVIOUS WORK

The structure of sterane was formed on the early stages of natural selection of the organisms. In the steroid aspect this structure is a “comfortable” bioorganic frame for arranging different functional groups and radicals. Definite combination and topography of these groups and radicals determines the formation of a great number of compounds with various, especially, hormone activity of different types. As per their structure brassinosteroids are close to steroid hormones of animals, they have specific influence on the cell membrane permeability, regulating the inflow of definite class of substances, i.e. they increase permeability for some substances and prevent others from penetrating. The second mechanism of influence is specific impact on the activity of definite enzymes (biocatalysts,

ferments), regulating the metabolic process and functions of the cells, through the system of adenylate cyclasa. And finally the above system influences directly the functions of the cells genome providing for the possibility of new genes expression revealing genes which were silent before. It also makes possible the emergence of proteins supporting new functions which lacked before [2]. Concentrated in the growth segments of browses and pollen, brassinosteriods penetrate the organisms of invertebrates and vertebrates, causing different specific and general protective effects. The content of brassinosteroids in plants is very low, hence the chemical synthesis is the only source of such compounds in substantial amounts.

## 3. RESULTS

We have been carrying out the researches of brassinosteriod properties since 1994. Currently we are engaged in researches on a number of perspective directions in the Laboratory of Biotechnologies located in ASU Technology Park (Astrakhan Region, Privolzhsky District, settlement Nachalo). We are carrying out researches on impact and development of original preparations on the basis of perspective biologically active substances (BAS) to increase the survival rate of valuable biological objects, as well as on implementation of nanoregulators in biological systems vital processes management. Preparations on the basis of brassinosteroids possess a wide range of stimulatory and protective properties and thus increase the yield and quality of the agricultural products. Epin-extra preparation, that is known in Russia and abroad, with epibrassinolide as active substance, was synthesized at the Chemical Faculty of Moscow State University and developed as a ready-to-use drug by the colleagues from LLC Non-profit Production Partnership "NEST M". This preparation is an effective immune response modifier increasing the resistance of plants to stress and phytopathogenes. These effects are accomplished by initiating a sequence of biochemical transformations, such as activation and suppression of key enzymatic reactions, stimulation of protein synthesis, activation of photosynthesis etc. [3]. Epibrassinolide, active substance of the preparation, is a natural biological regulator which is traditionally present in animal and human nutrition and is metabolized in an evolutionally habitual way. It is an important guarantee of ecological safety of the preparation and it is proved by its toxicological testing. Furthermore the stimulation of plant natural resources by the preparation allows diminishing the usage of other protective means for plants. Preparations on the basis of brassinosteroids can be regarded as a new generation of environmental friendly agricultural specimens with natural way of impact and measuring that is comparable to their contents in the natural conditions. Researches on the impact of brassinosteroids on animals haven't been carried out before.

## CONCLUSION

We were the first to carry out complex researches on the influence of epibrassinolide on animals, microorganisms of different level of phylogenesis icluding the ontogenesis aspect [3; 4; 5]. Analyses of lipid peroxidation (LPO), permeability of blood-tissue interface (PBTI) as well as some other biochemical parameters of blood prove the absence of real pathologic

changes and evidences in case of epibrassinolide inflow into the organism of the vertebrates of different classes during the early stages of ontogenesis [5]. Epibrassinolide promotes the stabilization of wide range of the vertebrate physiological parameters during the early stages of ontogenesis and leveling them to the control level in case the vertebrate specimen is in adverse conditions of the environment and is exposed to the experimental effect of toxic substances of different types. We have detected some effects of brassinosteroids on animals, plant bodies and microorganisms permitting to class the impact of epibrassinolide as substance intensifying antioxidant processes in the organisms of different level of phylogenesis [2; 6; 7].

Currently we are developing new biological preparations to increase the survival rate of valuable target species, aquarium biological objects and valuable objects of aquaculture [2; 6].

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*Chapter 17*

# THE POSSIBILITY OF USE OF EXTRACTS FROM HIGHER HYDROPHYTES AND GEOPHYTES OF THE ASTRAKHAN REGION FOR THE NEEDS OF COSMETOLOGY

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## ABSTRACT

A current concern of modern medicine, pharmacology and cosmetology is the replacement of chemical antimicrobials by natural herbal ingredients. Some of these components are biologically active substances in plant extracts. These are concentrated extracts from plant materials. The effectiveness of these extracts is largely due to the presence of certain chemicals. These existing active substances have a diverse composition and belong to different classes of chemical compounds: flavonoids, terpenoids, glycosides, some saponins, resins, phenolics, volatile compounds and tannins, carbohydrates and minerals.

The unique composition of complex and biologically active substances in fresh-water hydrophytes and geophytes indicates a wide range of application. These substances attract the particular interest of specialists in the field of cosmetology.

We are currently studying a selection of individual components from the test extracts of plants. We will plan to use these substances in the preparation of cosmetic products.

**Keywords:** Biologically active substances, antimicrobial activity, dermato-protective activity, extracts of plants, biotechnology, cosmetics, higher hydrophytes, geophytes, thalassotherapy

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## 1. INTRODUCTION

Currently, the market for medicines and cosmetics in Russia based on plant raw materials occupy a rather modest place compared with synthetic drugs (talking about finished products based on plant materials) [11]. However, the need of the population in the natural products based on plant raw materials is not currently satisfied mainly due to the lack of quality of medicinal plant materials. The range and volume of offerings on the market of medicinal plant materials is below current needs, the growth of which is has been noted in recent years [12].

With industrial production total phyto efficiency of extraction of biologically active substances, in some cases reaches only 40-50% due to insufficient depletion of cake for all groups of active substances [12].

This indicates the need to improve and develop new advanced technologies resource complex processing of medicinal plant materials, providing maximum extraction of bioactive substances.

In addition, a current concern of modern medicine, pharmacology and cosmetology is the replacement of chemical antimicrobials by natural herbal ingredients. Some of these components are biologically active substances in plant extracts - concentrated extracts from plant materials. The effectiveness of these extracts is largely due to the presence of certain chemicals. These existing active substances have diverse composition and belong to different classes of chemical compounds.

The experimental data of many domestic and foreign researchers characterize hydrophytes as a unique natural renewable source of a number of important compounds which can be used in industry. The chemical composition of marine and freshwater plants depends on the type, stage of development and growth conditions. Hydrophytes of the Volga-Caspian are a very valuable raw material for the production of products which can be used for various purposes depending upon their biochemical composition. The composition of an organic substance includes of cellulose, nitrogenous compounds, soluble in alcohol (chlorophyll), and ether-soluble substances, polysaccharides, vitamins A (carotene), B, and micro-elements like magnesium, zinc, iron, cobalt, titanium.

Benefits of biotechnology that are relevant to plants is to produce products with well-defined chemical composition, which, in turn, were also obtained from plants, but under the influence of enzyme catalysts. As a result, new and valuable natural biologically compatible material can be created. Today the possibilities of biotechnology are endless.

These researches are carried out in Astrakhan State University (ASU) Biotechnologies laboratory (Russia, Astrakhan region, Privolzhsky district, Nachalo village) with the collaboration of international the specialists at Ca'Foscari University (Venezia, Italy).

## 2. PURPOSE AND RESEARCH METHODS

The object of our study to identify the active chemical ingredients in plants of the Astrakhan region, as well as to research methods of extracting biologically active substances from plants, both water and land, and to apply the results in the field of cosmetics, including thalassotherapy.

The direction of this study - selection (creation) and use of plant extracts for cosmetic purposes - is a both new and relevant to the Astrakhan region in particular. The originality and novelty of the isolation and production of biologically active extracts with anti-microbial properties in plants ecologically safe areas of the Astrakhan region lies in the fact that natural environmental conditions of high insolation, high temperatures and low humidity contribute to the formation of biologically active substances with higher concentrations.

The uniqueness of the Astrakhan region lies in all the favorable environmental conditions (special climatic, hydrological and biotic factors) which allow for the accumulation of wild plants with a full range of biologically active substances, and which is, undoubtedly, the determining factor in the creation of original herbal remedies for the needs of cosmetology at a much higher antimicrobial activity compared with the known properties of similar plants in other regions of the Russian Federation. In addition, the plants which grow here, accumulate a large number of different chemical composition of biologically active substances. This BAS determines the antimicrobial, bactericidal, immuno-protective and anti-TB activity of extracts (tinctures, decoctions, etc.) from plant material.

We studied the methods of extracting pectines from the hydrophytes of the Northern Caspian sea and the Volga river delta, such as perfoliate pondweed (*Potamogeton perfoliatus L.*) [14] and the methods of extracting biologically active components (such as flavonoids from the kidney of black poplar *Populus nigra*, flavonoid compounds of licorice's roots *Glycyrrhiza glabra* and inflorescences' everlasting *Helichrysum arenarium L*) [17].

Samples of hydrophytes were removed from the water, rinsed and dried in natural conditions and kept at a relative humidity of 75% and had a residual water content of 10 - 12% to extract a pectin-similar substance (rdestin).

In the first stage of the experiment it was found the content of impurities in the freshwater plant perfoliate pondweed, studied the organoleptic and physico-chemical quality of water plants. This was followed by the sanitary-hygienic assessment of freshwater plants. Next we studied the chemical composition of raw (samples of freshwater pondweed), which showed the potential for processing in order to obtain extracts.

The molecule rdestin includes carboxyl groups which are responsible for its ability to form salt - rdestats can be used in food, confectionery industry, cosmetology, pharmaceutical industry [16].

Also we studied the antimicrobial properties of extracts of plants of the Astrakhan region, as well as the possibility of using the extracted active substances these plants for antimicrobial cosmetics and other products for example, the use of a combination of extracts from plants (licorice naked, yarrow, H. arenarium) in preparation of balms for skin problems (cracks, scratches, dryness, burning, etc.).

In addition, we investigated the antimicrobial activity of hydroalcoholic and aqueous extracts from the kidney of black poplar (*Populus nigra*), inflorescence of H. arenarium (*Helichrysum arenarium L.*), yarrow (*Achillea micranta L.*), licorice root naked (*Glycyrrhiza glabra*) relative to strains of *Staphylococcus aureus* (DSM 6538799) and *Staphylococcus aureus* which was isolated from the environment (from the skin, the microflora of water) [9].

Research in the activity of extracts of these plants in experiments show a direct effect on the suspension of *Staphylococcus aureus* in culture. Control of *Staphylococcus aureus* was shown on the surface of the medium IPA or ACI in Petri dishes in a bar segmental method under sterile conditions [7].

At this stage of the experiment is carried out the selection of individual components of the studied extracts of higher water plants of the Volga river and terrestrial plants of the Astrakhan region, and planned their integrated use in the preparation of cosmetic products for various purposes.

## 3. RESULTS

The results of experiments to determine the chemical and carbohydrate composition suggest that the water content in air-dry plant pondweed *Potamogeton perfoliatus L.* is about 9.3 %. Dry matter freshwater grass (90.7 %) consists of organic materials: ash (20.4 %), crude protein, soluble and insoluble carbohydrates.

As part of perfoliate pondweed contains large amounts of carbohydrates, including in particular tissue – 19.7 %, and pectin – 18.4 %, as well as easily-hydrolysable and soluble carbohydrates in an amount of 11.2 % and 9.6 % respectively. Contents in the ether-soluble substances is about 1.7 % of total nitrogen in dry matter is negligible (7.5 %).

Thus, high levels of carbohydrates and minerals, as well as microbiological safety perfoliate pondweed indicates the possibility of using water plants as raw material for extracts [16].

Research activity of extracts of these plants in the experiments show a direct effect on the suspension of *Staphylococcus aureus* in culture.

Highest antimicrobial activity against a *Staphylococcus aureus*, isolated from the external environment, have kidney extracts of black poplar, since as a result of the hydroalcoholic extraction of flavonoids are allocated, in particular pinotsembrin and pinostrobin and whose presence and cause a high antimicrobial activity of extracts.

The greatest antimicrobial action has extracts of poplar buds black - when you make them in IPA almost complete suppression of significant growth and development of colonies of *Staphylococcus aureus* in comparison with single colonies under the influence of other extracts.

Antimicrobial activity of extracts of other plants in relation to *Staphylococcus aureus* is due to release from the original extraction of flavonoids and flavones, including extracts of *Glycyrrhiza glabra* - naringenin, quercetin, kempfelola, *Achillea micranta* and Helichrysum *arenarium* - naringenin, quercetin, azulene, etc. [7].

An examination of the chemical components of the extracts of various plants used in this report by thin-layer chromatography, gas chromatography / mass spectrometry showed, according to other studies and the literature, in an extract of the roots and rhizomes of *Glycyrrhiza glabra* the presence of glucopyranoside, dioksiflavonon, naringenin, parengenina, caffeine and other ingredients; inflorescences *Helichrysum arenarium*, *Achillea micranta* the presence of quercetin, azulene, kaempferol, and others; and in extracts of *Populus nigra*, according to published data, the presence of pinotsembrina and pinostrobina, according to experimental data in the presence of asarone in aire marsh (*Acorus calamus*) [1,2,3,5,6,10,15].

## CONCLUSION

Today, more attention is paid to the industrial use of carbohydrates water plants (cellulose, cellulose, pentosans, pectins).

Entire lines of beauty products based on extracts of water plants are being developed and, naturally, they are used for direct contact with the human body through the wraps. The use of algae for cosmetic purposes is called thalassotherapy (from the Greek "Thalasso" - sea). Wrapping has a beneficial effect on the human body with through the integrated use of the gifts of the sea - algae, salts, seawater, and sea mud which is rich in minerals and other trace elements.

Currently, thalassotherapy, which has several areas, is one of the most popular types of health and beauty treatment in the world.

Unfortunately, in our country for various reasons, this trend did not develop in our health resorts. Therefore, we are lagging behind Western European countries, although have all the necessary facilities and conditions for success. First of all, this is the output of many seas, including the Caspian sea, the presence of research institutes and laboratories.

The possibility of using the extracts of terrestrial plants which we have studied in cosmetic products, in particular, for the production of antimicrobial cosmetics with aseptic properties, as well as the use of extracts of water plants for the preparation of cosmetics used in thalassotherapy.

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*Chapter 18*

# ANTIOXIDANTIVE ACTIVITY OF DRY AND GERMINATED SEEDS OF AGRICULTURAL CULTURES

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## ABSTRACT

Determination of the total content of endogenous antioxidants (AO) in seeds of the investigated cultures and sprouts was performed by an express method of initiated oxidation of hydrocarbon – cumene developed in the Institute of Chemical Physics RAS. The concentration of vitamin C in dry seeds and sprouts was obtained by a liquid-chromatographic method. These were found to lie in the range $1.1 \cdot 10^{-6} – 1.1 \cdot 10^{-5}$ M/kg in seeds. In sprouts of these cultures the range was $1.8 \cdot 10^{-5} – 1.1 \cdot 10^{-4}$ M/kg. The maximum content of endogenous AO in the sprout material of investigated cultures was $1.4 \cdot 10^{-2} – 2.1 \cdot 10^{-1}$ M/kg.

**Keywords:** Grain cultures, germinated seeds, antioxidants (AO), oxidation

## INTRODUCTION

The process of seed germination is unique and consists of a complex of biochemical transformations. The soil in which the seed is germinating has a large number of microorganisms that constitute a good nutrient medium for the swelling seed. But many negative forces, such as contaminated soil, water and air act on the seed. This results in the formation of excessive free radicals. A system of AO protection is induced in the seed to counteract the damaging action of these factors. The AO are regulators of plant growth. In the process of germinating, complex reserve matter of the seed breaks up and is then easily

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assimilated. The amount of AO (vitamins, bioflavonoids) and microelements is significantly increased in the germinating seed. All useful matter in the living tissue is contained in optimal amounts and proportions. For analysis purposes, the following seeds and sprouts were taken: grain cultures - wheat, rye, oats; bean cultures – lentils, "Nut" peas and "Masch" peas; buckwheat, pumpkin, sunflower, sesame, flax, milk thistle. The purpose of our investigation was to perform a test on the content of biologically active substance (BAS), i.e., to determine the total content of endogenous AO in seeds of the aforementioned cultures and sprouts at various stages of growth.

**Table 1. Data on Vitamin C in Dry and Germinated Seeds At Maximum Stages of Growth**

| № | Type of Culture | Vitamin C Concentration in Dry Seeds (M/kg) | Vitamin C Concentration in Germinated Seeds (M/kg) |
|---|---|---|---|
| 1 | Oats | $2.0 \cdot 10^{-6}$ | $4.2 \cdot 10^{-5}$ |
| 2 | Rye | $1.1 \cdot 10^{-6}$ | $2.6 \cdot 10^{-5}$ |
| 3 | Wheat | $2.0 \cdot 10^{-6}$ | $1.8 \cdot 10^{-5}$ |
| 4 | Lentils | $4.9 \cdot 10^{-6}$ | $1.1 \cdot 10^{-4}$ |
| 5 | Peas "Masch" | $1.1 \cdot 10^{-5}$ | $7.5 \cdot 10^{-5}$ |
| 6 | Peas "Nut" | $3.5 \cdot 10^{-6}$ | $6.4 \cdot 10^{-5}$ |
| 7 | Buckwheat | $2.6 \cdot 10^{-6}$ | $4.6 \cdot 10^{-5}$ |
| 8 | Sunflower | $2.8 \cdot 10^{-6}$ | $2.6 \cdot 10^{-5}$ |
| 9 | Pumpkin | $4.8 \cdot 10^{-6}$ | $5.5 \cdot 10^{-5}$ |
| 10 | Flax | $2.5 \cdot 10^{-6}$ | $4.0 \cdot 10^{-5}$ |
| 11 | Sesame | $3.9 \cdot 10^{-6}$ | $6.1 \cdot 10^{-5}$ |
| 12 | Milk Thistle | $3.5 \cdot 10^{-6}$ | $8.6 \cdot 10^{-5}$ |

The destructive action of free radicals, as is known, is neutralized by AO such as vitamin C. In all cultures that we have investigated, vitamin C is actively synthesized in the process of seed germination. We determined its amount in germinating seed during the first ten days by a liquid chromatographic method. The obtained results are presented in a table and diagram.

*Diagram Illustrating Results in Table 1*

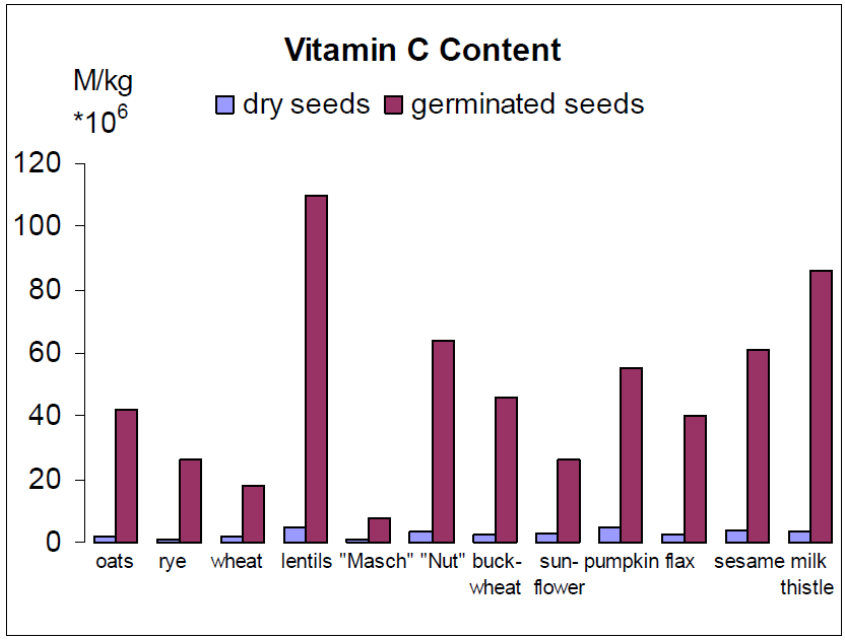
In comparing the intensity of the process of synthesizing vitamin C in germinating seeds, one can say that it proceeds rapidly in oats, rye and wheat.

In bean cultures, first is lentils, then "Masch" peas and "Nut" peas.

In Milk Thistle, the main antioxidant being bioflavonoid, the amount of vitamin C increases for eight days.

Synthesis of vitamin C for most cultures increases intensively from the fifth to the eighth day.

The property of germinating seeds to intensively synthesize vitamin C may be a general property of highly evolved plants.




## METHOD OF EXPERIMENT

Determination of the total content of endogenous antioxidants (AO) in seeds of the aforementioned cultures and sprouts was performed by an express method of initiated oxidation of hydrocarbon - cumene, developed in the Institute of Chemical Physics RAS. It enables to determine the mechanism of antioxidants (AO) of complex composition, their concentration and the inhibitor constant ($K_7$). The method is based on the laws of a chain free-radical oxidation mechanism, when matter containing AO introduced in a model reaction reacts with an active center (peroxide radical $RO_2$). The reaction slows down due to disruption of the chain reaction when the AO reacts with $RO_2$. The induction period ($\tau$) can be determined from the braking time of the oxidation reaction and the AO concentration in the investigated preparation is calculated. The period of induction $\tau$ and the quantity of oxygen consumed $\Delta O_2$ are determined from the following formulas /1-3/.

$$\tau = \frac{2[InH]_0}{Wi} \quad (1)$$

$$\frac{\Delta O_2}{[RH]} = -\frac{k_3}{k_7} \ln (1 - t/\tau) \quad (2)$$

$W_i$ - the initiating rate, is calculated from the formula

$$W_i = 6.8 \cdot 10^{-8} [AIBN] \text{ mole} / l \cdot s , \quad (3)$$

where [AIBN] (AZO-bis-ISOBUTYRONITRILE) is the initiator concentration in mg per ml of hydrocarbon; RH - hydrocarbon and InH - antioxidant.

The concentration of analyzed antioxidant $[InH]_0$ is calculated from expression (1) using the experimentally determined induction period $\tau$ and the known initiation rate $W_i$. The inhibiting rate constant $k_7$, determining the anti-radical activity of the antioxidant and constituting its quality characteristic, is found from relation (2) using the known constant of the chain continuation rate $k_3$, hydrocarbon concentration [RH], experimentally determined induction period $\tau$ and the quantity of absorbed oxygen $\Delta O_2$. The greater the amount of AO in the sample the greater the period of induction $\tau$.

A detailed description of the method can be found in [1-4].

## RESULTS AND DISCUSSION

Experimental data enabled to obtain the total AO content of the examined samples. As an example, the figure shows the kinetic dependence of absorbed oxygen in a reaction of cumene initiated oxidation (straight line 1) and the presence of dry seeds of sesame, wheat, "Nut" peas and milk thistle (curves 2-5). It can be seen from the figure that, in the absence of the additive, hydrocarbon oxidation proceeds at constant rate (straight line 1). Upon introducing seeds, the oxidation rate at the initial instant is greatly retarded and begins to increase after some time, which indicates that there is AO in the additive. The growth of the reaction rate is due to the expenditure of AO. After its expenditure, the reaction reaches a constant non-inhibitor reaction rate. The time of AO expenditure ($\tau$) is determined graphically on the kinetic curve by the point of intersection of two straight lines, one of which is the straight-line portion of the kinetic curve after AO has been used up. The other is the tangent to the kinetic curve whose inclination angle is one-half the tangent angle of the first. Data on analysis of AO are present in the Table 2.

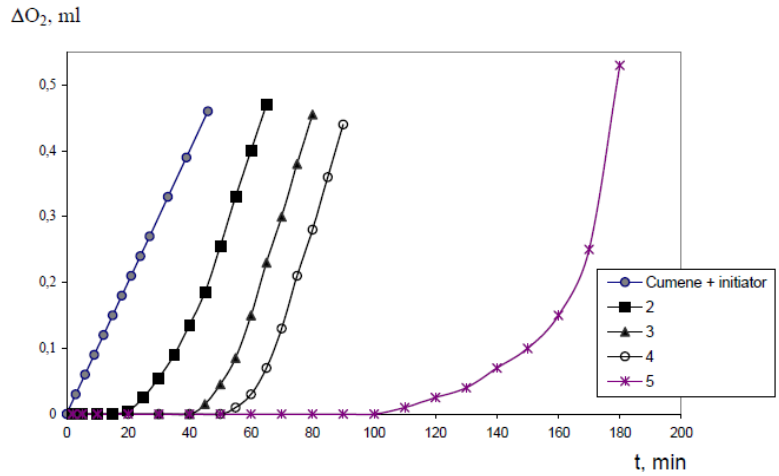



Figure 1. Kinetic Dependences of Oxygen Absorption. 1 – hydrocarbon (cumene) + initiator (AZO-bis-ISOBUTYRONITRILE, 2 mg), 2 – with sesame added (15.3 mg), $\tau$ = 24.5 min, 3 – with wheat added (91 mg), $\tau$ = 51 min. 4 – with "Nut" peas added (33 mg), $\tau$ = 63 min. 5 – with milk thistle added (78 mg), $\tau$ = 167 min. Hydrocarbon 1 ml, t = $60^0$ C.

**Table 2. The Total Content of Endogenous AO in Dry and Germinated Seeds**

| № | Type of Culture | AO Concentration in Dry Seeds (M/kg) | AO Concentration in Germinated Seeds (M/kg) |
|---|---|---|---|
| 1 | Oats | $3.8 \cdot 10^{-3}$ | $8.0 \cdot 10^{-2}$ |
| 2 | Rye | $1.6 \cdot 10^{-3}$ | $3.8 \cdot 10^{-2}$ |
| 3 | Wheat | $3.4 \cdot 10^{-3}$ | $3.1 \cdot 10^{-2}$ |
| 4 | Lentils | $5.4 \cdot 10^{-3}$ | $3.1 \cdot 10^{-2}$ |
| 5 | “Masch” Peas | $7.8 \cdot 10^{-3}$ | $5.2 \cdot 10^{-2}$ |
| 6 | “Nut” Peas | $6.7 \cdot 10^{-3}$ | $1.2 \cdot 10^{-1}$ |
| 7 | Buckwheat | $1.0 \cdot 10^{-3}$ | $1.7 \cdot 10^{-2}$ |
| 8 | Sunflower | $1.6 \cdot 10^{-3}$ | $1.4 \cdot 10^{-2}$ |
| 9 | Pumpkin | $1.2 \cdot 10^{-3}$ | $1.4 \cdot 10^{-2}$ |
| 10 | Flax | $1.2 \cdot 10^{-3}$ | $7.6 \cdot 10^{-2}$ |
| 11 | Sesame | $6.5 \cdot 10^{-3}$ | $9.8 \cdot 10^{-2}$ |
| 12 | Milk Thistle | $8.7 \cdot 10^{-3}$ | $2.1 \cdot 10^{-1}$ |

In addition to a general positive effect on the human organism germinating seeds of each individual culture have specific properties that make them useful for those suffering from definite ailments.

Thus, the germinating seeds of cereals normalize the functioning of the digestive system by their action on stomach microflora. The germinating seeds of wheat and rye, containing group B and E vitamins that regulate exchange processes, promote normal functioning of the brain and heart hinders the formation of blood clots, decreases the consequences of stress situations and improves skin and hair condition.

Lentils germinating seeds promote blood creation, necessary in fighting colds.

Buckwheat germinating seeds improve the condition of blood vessels since they are a good source of rutin (antisclerotic vitamin) that strengthens their walls, decreases the penetrability and breakage of capillaries, hinders hemorrhaging in the retina and increases the level of hemoglobin.

Pumpkin germinating seeds have a vermifuge action, useful in treating the prostate, improve functioning of the brain, and have a beneficial effect on the reproductive system and the urino-genital system.

Sunflower germinating seeds, containing vitamin D, fortifying the nervous system, promote retention of good vision and improve skin condition.

Sesame germinating seeds strengthen bones, teeth and nails, promote prophylaxis of osteoporosis, and are recommended when fracturing bones, especially useful for pregnant women and breast-feeding mothers for the large amounts of calcium and magnesium contained in them and for children during the period of intensive growth and changing teeth.

Flax germinating seeds contain a large amount of linolic acid which participates in the formation of the cellular membrane, restores the elasticity and firmness of the blood vessels, supports functioning of the brain, helps to solve menopause problems for women and various disturbances of the digestive system. Milk thistle germinating seeds are a powerful hepatic protector, reduce the destructive aspects of medicinal preparations and toxic substances entering the human organism from the environment and are effective in treating hepatitis C.

Clinical tests were performed on using germinating seeds of various cultures and clearly positive results were obtained. Regular consumption of germinating seeds stimulates metabolism and blood creation, improves immunity, compensates vitamin and mineral deficiency, normalizes acidic-alkaline balance, promotes digestion and retards aging. It was established from these investigations that in the analyzed germinated seeds the AO concentration varied within the limits $1.4 \cdot 10^{-2} - 2.1 \cdot 10^{-1}$ M/kg. This relatively high AO concentration is comparable to the values of AO activity for dry medicinal and spice-aromatic plants: cloves, Oleoresin of nutmeg, coriander, oregano, Oleoresin of black pepper.

Germinating seeds of wheat, rye and oats are widely used for health-building in diets for athletes and others in many European countries.

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*Chapter 19*

# FILAMENTOUS FUNGI OF WASTE BIOMASS OF HIGHER WATER PLANTS

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## ABSTRACT

Filamentous fungi mainly represented by species of *Aspergillus* and *Penicillium* genera were isolated from waste biomass of higher water plants (HWP). It was established that density of *Aspergillus* species isolated from biomass of HWP cultivated on sewages was 2-3 times higher compared to biomass of HWP cultivated on fresh water. Micromycetes possessing cellulase activity and natural resistance to certain pollutants were determined.

**Keywords:** Higher water plants, biomass, pollutants, cellulase activity, filamentous fungi

Uzbekistan possesses certain resource potential of renewable energy sources, which may be used for biofuel production. Among many plant resources a separate place is taken by cleansing facilities with waste plant biomass produced by higher water plants (HWP) – water lettuce (*Pistia stratiotes L*.) and water lily (*Eichhornia crassipes* Solms.), which are used in the biotechnology of sewages cleansing developed at the Institute of microbiology (Tashkent, Uzbekistan) [1]. Industrial development is inevitably linked with the problem of accumulation of wastes and development of efficient methods for cleansing the environment from different pollutants. In Uzbekistan this biotechnology is applied in oil-and-gas and food industries. During the process of pollutants transformation a huge quantity of waste plant biomass is accumulated on territory of cleansing facilities. Due to high content of pollutants in that biomass it cannot be utilized for agricultural needs.

Cleansing in this system takes place on account of following components:

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1. Association of microorganisms and HWP. HWP possess extremely developed root system, which hold certain portion of biogens (nitrogen, phosphorus) and pollutants incoming to ecosystem from sewages, and serves as natural immobilizer for microorganisms that participate in transformation of pollutants.
2. Benthos, holding and absorbing part of biogens and pollutants migrating on border of division water/bottom deposits.
3. Microorganisms.

Thus, the more pollutants were in sewages the more pollutants are absorbed by HWP biomass, which will create extreme conditions for development of microorganisms.

It is necessary to note that HWP are characterized by valuable biofuel properties and high productivity. Water lettuce *Pistia stratiotes* cultivated on sewages gives more than 1 kg of biomass growth from 1 $m^2$ of water surface daily, which makes 90-135 tons of dry biomass from 1 hectare of water surface per season. HWP applied for sewage cleansing contain protein, cellulose, oils, water soluble polysaccharides. That is why waster biomass of HWP represents certain interest for its transformation into biofuel (biogas, bioethanol). Isolation of microorganisms from natural biocenosis, which are capable to degrade cellulose, and determination of optimal cultivation conditions for their utilization of substrates containing certain pollutants is perspective approach for biofuel production.

We conducted search and isolation of micromycetes from waste HWP biomass that possess cellulase activity and natural resistance to certain pollutants.

For that purpose we used biomass of HWP cultivated on sewages of oil-and-gas industry. Czapek-Dox and modified Czapek-Dox media were used for isolation of micromycetes by method of subsequent dilutions [2]. Reduced sugars wre determined according to Somogyi-Nelson [3]. Incubation of micromycetes was conducted 6-7 days. Taxonomy of micromycetes was determined according corresponding manuals [4, 5].

As result of conducted study it was established that in process of sewages cleansing with application of association of HWP and microorganisms not only total quantity of microorganisms isolated from biomass raised but their taxonomic structure changed as well.

Phenomenon of "domination concentration" is well known for macroorganisms in stress conditions. Similar pattern in reaction of micromycetes was observed at analysis of rotten rice, where *Penicillium*, *Aspergillus* and *Stachybotrys* were prevailing species among isolated fungal cultures [6]. We observed same type of reactions on pollution of water reservoirs at analysis of waste HWP biomass, which in this case serves as immobilizer not only for microorganisms but for a number of pollutants as well.

At stress conditions of pollution certain groups of micromycetes prevailed with increased concentration of pollutants in sewage. Filamentous fungi isolated from waste biomass belonged mainly to genera *Aspergillus* and *Penicillium.* At the same time density of *Aspergillus* species raised by 2-3 times compared to number of *Aspergillus* species isolated from biomass cultivated in fresh water (figure 1), whereas prevailing fungi on waste biomass from fresh water belonged to *Fusarium* genus.

Different cultures of filamentous fungi were isolated from waste HWP biomass, which were studied for ability to degrade cellulose. According to cellulase active following cultures were selected: *Aspergillus niger, A. terreus, A. flavus, Penicicillium griseo-roseum.* These cultures expressed good growth on Mandels medium with filter paper and on modified Czapek-Dox medium with addition of cellulose substrate.

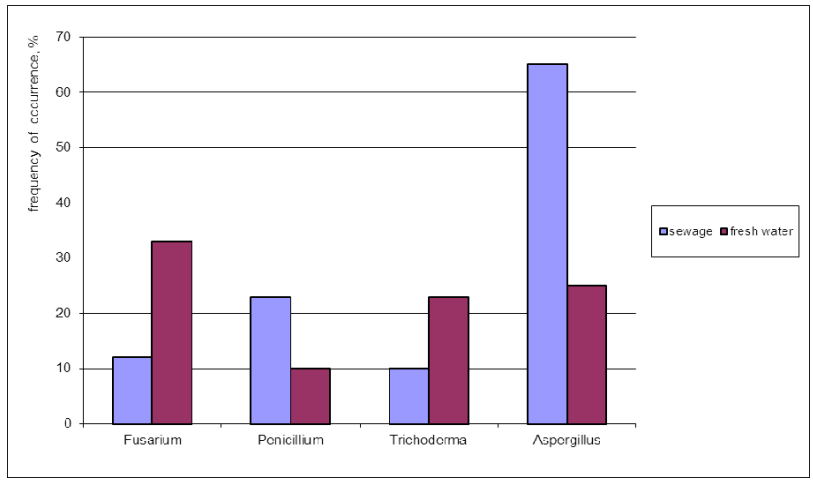


Figure 1. Frequency of occurrence of different micromycetes.

Peculiarities of cellulose degradation by filamentous fungi are linked with such factors like cellulose structure and genesis, which determine to considerable extent the specificity of cellulose digestion. There are numerous data about influence of cultivation conditions on cellulase activity. It is necessary to note quality and quantity of used sources of nitrogen and carbon among main factors impacting biosynthesis of cellulase. It is necessary to develop such medium for cultivation of fungi-producers of cellulase that would secure biomass accumulation and high enzymatic biosynthesis.

**Table 1. Comparative activity of formation of endoglucanase at micromycetes cultivation on Mandels and Farid media**

| № | Variants | Endoglucanase activity, U/ml | |
|---|---|---|---|
| | | Farid medium + filter paper | Mandels medium + filter paper |
| 1 | *Aspergillus niger* | 1.6 | 2.4 |
| 2 | Aspergillus terreus | 3.8 | 4.8 |
| 3 | *Aspergillus flavus* | 1.6 | 2.2 |
| 4 | *Penicicillium griseo-roseum* | 2.4 | 4.3 |

**Table 2. Comparative activity of formation of endoglucanase at micromycetes cultivation on Mandels and Farid media with addition of HWP biomass**

| № | Variants | Endoglucanase activity, U/ml | |
|---|---|---|---|
| | | Farid medium + 2% pistia | Mandels medium + 2% pistia |
| 1 | *Aspergillus niger* | 2.7 | 3.9 |
| 2 | Aspergillus terreus | 9.2 | 9.5 |
| 3 | *Aspergillus flavus* | 2.6 | 3.1 |
| 4 | *Penicicillium griseo-roseum* | 3.9 | 4.5 |

We studied endoglucanase activity of filamentous fungi cultivated on Mandels and Farid media with filter paper in comparative aspect (table 1). Farid medium earlier was successfully used for some protoplast cultures of micromycetes [7]. It is optimal for submerged cultivation and production of cellulolytic enzymes.

Determination of endoglucanase activity is the most specific and precise index for our needs. The maximum endoglucanase activity was observed at strain *Aspergillus terreus.* Cultivation of micromycetes revealed that in all variants the stable increase of endoglucanase activity is observed on media with natural substrates (waste HWP biomass) (table 2).

Study of development of microorganisms on nutrient media with waste HWP biomass revealed that presence in medium of organic nitrogen shortens lag-phase at selected producers of cellulase and induces increased biosynthetic activity of filamentous fungi, which allows to reach maximum indices on $4^{th}$ day of cultivation.

Based on preliminary evaluation of cellulolytic properties of selected cultures it may be assumed that the higher spectrum of cellulase enzymes is probably characteristic for them. Obtained results testify possibility of receipt of active cultures of micromycetes for application in biofuel production.

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*Chapter 20*

# PURIFICATION OF KAOLIN OF DIFFERENT GRADES WITH APPLICATION OF SILICATE MICROORGANISMS

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## ABSTRACT

Model tests on iron removal from primary kaolin of Angren deposit with application of Acidithiobacillus ferrooxidans K-1 and silicate microorganisms by two-stage scheme were conducted. Decrease of iron content in form FeO till 0.2% and $Fe_2O_3$ till 0.27% was established.

Application of microorganisms in purification processes of kaolin of AKS grade allowed to decrease content of $Fe_2O_3$ till 0.23% and to improve its technological properties.

**Keywords**: Kaolin, silicate microorganisms, Acidithiobacillus ferrooxidans, micromycetes, organic acids, iron oxides

Iron oxides present in kaolin, quartz sands and soil in form of impurities may considerably decrease quality of raw materials. Different non-biological methods are used to remove iron. But, some of these methods, like magnet separation and flotation, are not suitable for universal application and their efficiency to great extent depends on properties of ore materials to be processed.

Chemical methods based on minerals leaching by organic acid and treatment by reductant. They are usually used for removal of high quantity of iron; but, at the same time, they are more expensive, processing conditions are harder, and process is not environment friendly.

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Iron leaching from oxidized minerals with application of microorganisms is well known [1-3]. Numerous works revealed that rational and ecologically safe method of iron removal is application of iron-oxidizing microorganisms that transfer iron into soluble form and decrease its content till 0.7-0.9% in form $Fe_2O_3$. But, after treatment of kaolin by thionic iron-oxidizing bacteria the process of formation of quite strong iron hydroxide film of surface of minerals takes place.

Application of microorganisms-producers of organic acids allows to remove residual concentrations of iron. It is linked to formation of organic acids and other metabolites, which act as complex forming agents, and to enzymatic and non-enzymatic removal of iron as well [4-6]. Microorganisms-producers of mineral and organic acids impact minerals by increasing iron concentration in medium, which activates hydrolysis reaction and carry-over of metal cations.

The object of study was primary kaolin from Angren deposit and microorganisms Acidithiobacillus ferrooxidans. Model tests were conducted in Erlenmeyer flasks with ratio solid: liquid =1:7 with application of association of bacteria A. ferrooxidans K-1, initial pH of pulp was 1.5. Tests were conducted with partial change of liquid phase (up to 20%) by fresh nutrient medium. Preliminary cultivation of bacteria was conducted on medium K9 containing 6 g/l of $FeSO_4 \cdot 7H_2O$. Fresh nutrient medium with corresponding iron content was added accordingly. Cultivation period was limited by 2-3 days.

Conducted silicate analysis of cakes of bacterial leaching (BL) revealed that considerable decrease in concentration of FeO till 0.2% is observed, but, overprecipitation of oxide forms of iron is observed and, respectively, its increase, which negatively affects product quality. Further two day treatment of cake of BL by cultural broth of micromycetes allowed to decrease content of $Fe_2O_3$ till 0.27% (Table 1).

**Table 1. Results of chemical analysis of BL cake after treatment of primary kaolin by iron-oxidizing microorganisms *A.ferrooxidans* K-1 (1 stage) and silicate destroying microorganisms (2 stage)**

| Compounds | Content, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $SiO_2$ | $Fe_2O_3$ | FeO | $TiO_2$ | $Al_2O_3$ | CaO | $CO_2$ | $SO_3$ |
| Initial | 57.9 | 0.85 | 4.1 | 0.5 | 22.3 | 0.5 | 3.5 | 0.07 |
| 1 stage | 58.93 | 1.12 | <0.2 | 0.5 | 28.3 | <0.5 | <0.5 | 0.21 |
| 2 stage | 56.3 | 0.27 | <0.2 | 0.5 | 13.2 | <0.5 | <0.5 | 0.07 |

**Table 2. Results of chemical analysis of BL cake after treatment of AKS grade kaolin with cultural broth of micromycetes**

| Compounds | Content, % | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $SiO_2$ | $Fe_2O_3$ | FeO | $TiO_2$ | $Al_2O_3$ | CaO | $CO_2$ | $SO_3$ |
| Initial | 56.48 | 0.82 | 0.07 | 0.5 | 24.2 | 0.69 | 3.52 | 0.05 |
| After treatment with cultural broth | 52.5 | 0.23 | <0.2 | 0.5 | 13.2 | <0.5 | <0.5 | 0.07 |

It is necessary to note considerable decrease (almost in 2 times), on account of denial of application of expensive washing by diluted hydrochloric acid, volumes of discharge and washing solutions on all stages of processing.

Different microorganisms participate in degradation of main types of aluminum silicate minerals. These microorganisms are capable on account of synthesis of extracellular organic acids to transfer into soluble state compositions of calcium, potassium, titanium, silicon present in form of admixtures, which promotes to kaolin purification.

Destruction of silicate minerals is indirect process based on action of exometabolites produced by microorganisms in specific conditions of medium. To receive the maximum yield of acids it is necessary to strictly observe specific composition of medium and cultivation conditions. Production of organic acids by fungi demands on intensive aeration. It is reached by cultivation of micromycetes by surface method in vessels with thin layer of nutrient medium or by submerged method but in special bioreactors where aeration is conducted by mechanical stirring and insufflations of sterile air under pressure.

Selectivity and specificity of microbial destruction of silicate minerals is observed. This process depends on physiological peculiarities of microorganisms and formed by them organic and mineral acids, exopolysaccharides and other surface-active substances, and on type of silicate minerals and their crystal-chemical peculiarities as well.

Screening conducted among microorganisms on ability to synthesize organic acids allowed to select a number of microorganisms (Bacillus subtitlis var.mucilaginosus, Bacillus subtilis, Thrichoderma harzianum, Aspergillus niger). Di-, tri- and polycarboxilic acids, aminoacids, were determined in cultural broth of these microorganisms; and galactose and glucose was determined among exopolysaccharides. Maximum content of organic acids with prevailing content of citric acid was observed in cultural broth of Bacillus sp. (9.2 U), Aspergillus niger (13.4 U) and Thrichoderma harzianum (18.3 U).

We conducted model tests on kaolin bioleaching of AKS grade with application of selected microorganisms by single-stage scheme.

Preliminary cultivation of bacteria was conducted on experimental nutrient medium with addition of plant wastes. It is known that plant wastes are good substrate for cultivation of micromycetes. It results in production of organic acids, partial saccharification of substrate, production of polysaccharides, which presence promotes to improvement of plasticity of clays.

Kaolin of AKS grade was treated by filtrated solution of cultural broth at content solid: liquid =1:5, with presence of organic acids up to 25 U during 2 days.

Organic acids produced by these microorganisms in process of purification form complex links with elements incorporated in composition of crystal lattice of minerals, and thus, promote to minerals' dispersion. Obtained date testify that at treatment by cultural broth $Fe_2O_3$ content in kaolin decreases almost in 3 times and reaches 0.23% (table 2).

Received results reveal that treatment by bacterial solutions considerably improves quality of kaolin of AKS grade and increases level of its whiteness.

Thus, experimental results testify possibility of application of both thionic iron-oxidizing and microorganisms synthesizing organic acids for biopurification of kaolin of different grades.

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*Chapter 21*

# INFLUENCE OF VERBACTIN PREPARATION ON MICROBIOLOGICAL ACTIVITY OF IRRIGATED SEROZYON SOILS

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## ABSTRACT

Treatment of cotton seeds with Verbactin stimulated growth and development of useful soil borne bacteria enriching root system with active metabolites that positively impacted both seeds' germination, growth and development of cotton plants. It was determined that Verbactin activated community of soil borne microorganisms, mineralizing organic and mineral compounds, transformating of soil humus, including ammonificating and oligonitrophilic bacteria, Azotobacter, denitrifying bacteria, actynomycetes and filamentous fungi.

**Keywords:** Verbactin, Verticillium dahliae, microbial community, serozyom soil

Verticillium wilt on cotton is widely distributed and causes considerable losses to productivity of cotton all around the world [1-3]. One of the main approaches in improvement and stabilization of agricultural production is application of biological control of phytopathogens and pests [4, 5].

During last years in some regions of Uzbekistan an increased infection of cotton plants by Verticillium wilt caused by fungus *Verticillium dahliae* Kleb. is observed. The infection causes considerable losses to the yield and quality of fiber [6]. But, absence of domestic production of biopreparations and high cost of imported preparations, which are sometimes

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not effective in soil-climatic conditions of Uzbekistan, restrain application of biocontrol methods in Uzbekistan.

A complex biopreparation Verbactin to biocontrol Verticillium wilt on cotton was developed at the Institute of microbiology (Tashkent, Uzbekistan).laboratory, vegetative and field trials were conducted to evaluate influence of Verbactin on seeds' germination, growth, development, morbidity and yield of cotton. This work presents data on Verbactin influence on microbiological activity and resistance of cotton plants against Verticillium wilt on irrigated serozyom soils.

## METHODS OF STUDY

Tests of Verbactin efficiency were conducted in Tashkent region. Climate is characterized by high heat in summer; precipitation is mainly in winter and spring seasons; soil structure is loose, easily destroyable under treatment, irrigation and microorganisms' action. Content of humus is 1,025-1,042% and of gross nitrogen – 0,070-0,085%. Small plot ($25m^2$ each) trials were conducted on territory of Institute of cotton production. Soil is typical irrigated serozyom. Cotton variety Navruz was used in trials.

Verbactin preparation was applied two times: pre-sowing treatment of seeds and sprinkling of cotton plants before budding phase. Agrotechnics was common, according to agroclimatic conditions of the region. Every 12-14 days phenological observations were conducted to monitor growth and development of control (untreated) and test (Verbactin treated) plants. Soil samples from control and test plots were collected in three random spots.

Microbiological community was studied by cotton development phases: 2-4 leaves, budding, fruit formation, maturation. Microorganisms' content in rhizospheric soil was determined according to common microbiological methods with subsequent transfer on elective media (bacteria: ammonificating on beef extract peptone (BEP), spore producing on BEP and wort 7°B (v/v 1:1), digesting mineral sources of nitrogen on starch-ammoniac, oligonitrophilic and Azotobacter on Ashby, denitrifying on Giltay, nitrifying on Vinogradsky, aerobic cellulose degrading on Hutchinson, butyric-acid on Rushman; actynomycetes on oats and starch-ammoniac; fungi on Czapek). Quantity of microorganisms was recalculated per 1 g of absolutely dry soil. Bacteria, actynomycetes and fungi were isolated in each phase of cotton development from dominating colonies. Isolated cultures were purified and their taxonomy was determined according to common procedures [7, 8]. Antagonistic activity of microorganisms against *V. dahliae* was determined by method of agar blocks [9]. Observations on gradual lysis of sclerotia of fungus *Verticillium dahliae* 57 was conducted on spots of mycelium with high quantity of sclerotia by adding 50 µl of Verbactin in cylinders. The certain spot was selected and during 3 days monitoring of sclerotia lysis was conducted on microscope (Leica DM 1000; magnification x200).

## RESULTS AND DISCUSSION

Data of conducted analysis revealed that microbiological activity is more or less even, the quantity of microorganisms is not affected by sharp fluctuations depending on phases of

cotton development. Pre-sowing treatment of cotton seeds with Verbactin stimulated growth and development of useful microbiota, which enriched root system with active metabolites and positively affected growth and development of cotton in all phases of vegetation. Comparative analysis of dynamics of development of microbial community digesting organic and mineral nitrogen sources in test experiments (figure 1-3) revealed extremely positive influence of Verbactin on community of ammonificating bacteria. Number of ammonificating bacteria in typical serozyom soil under cotton changed from 3.8 to 9.2 x $10^6$ CFU/g soil. Their highest content was observed in phase of budding, with decrease in 2.8 times in phase of maturation. Interesting results were obtained in regards of quantity of bacteria digesting ammoniac forms of nitrogen and those that are responsible for disintegration of mineral nitrogen-free compounds. The ratio between quantity of bacteria growing on mineral and organic nitrogen reveals presence of mineralization process. Studied typical serozyom is characterized by high coefficient of mineralization. Thus, community of oligonitrophilic microorganisms is developed in typical serozyom soil; their highest quantity is observed in phase 2-4 leaves (1.81 x $10^7$ CFU/g soil). They play main role in nitrogen balance, and after their action serozyom is enriched by protein compounds that later are mineralized.

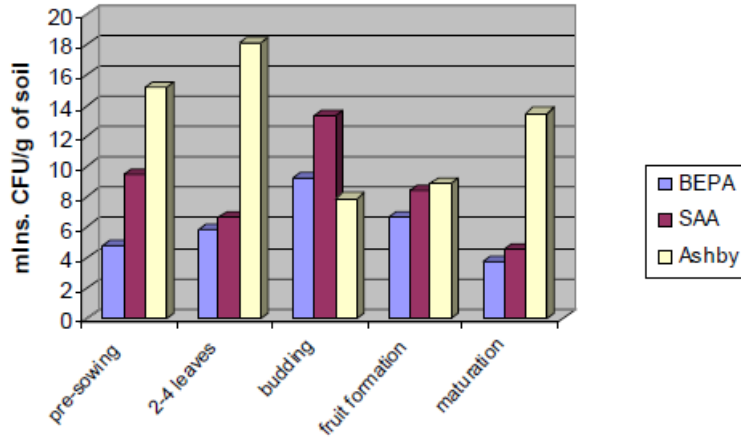



Figure 1. Influence of Verbactin on quantity of microorganisms digesting organic and mineral sources of nitrogen.

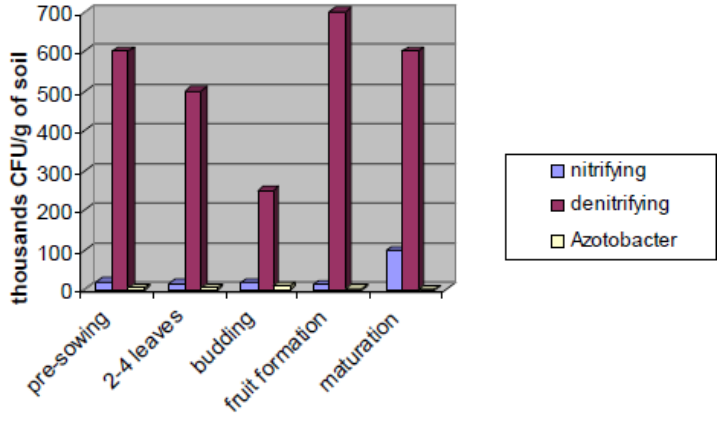



Figure 2. Influence of Verbactin on quantity of Azotobacter and nitrifying and denitrifying bacteria.

Simultaneously with ammonification the process of nitrification takes place in soil. But, in studied typical serozyom the number of nitrifying bacteria is low. Maximum of their development was observed in phase of maturation (1.2 x $10^5$ CFU/g soil). Quantity of denitrifying bacteria in test soil depending on phase of cotton development varied from 3.5 x $10^5$ to 7 x $10^5$ CFU/g of soil. Azotobacter content in serozyom soil did not change much and was within 3-10 x $10^3$ CFU/g soil.

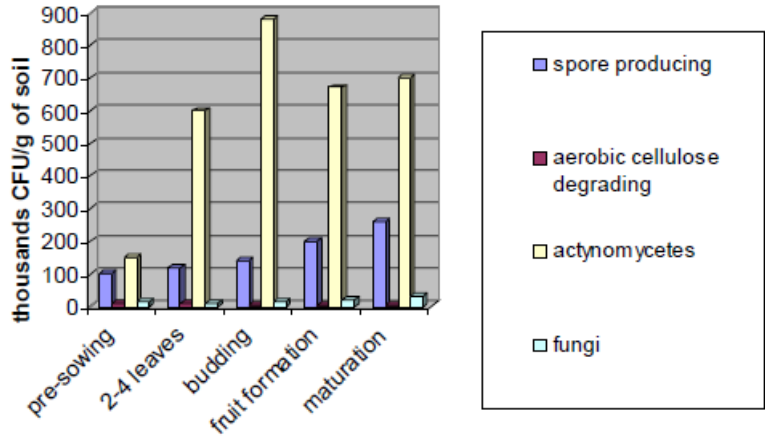
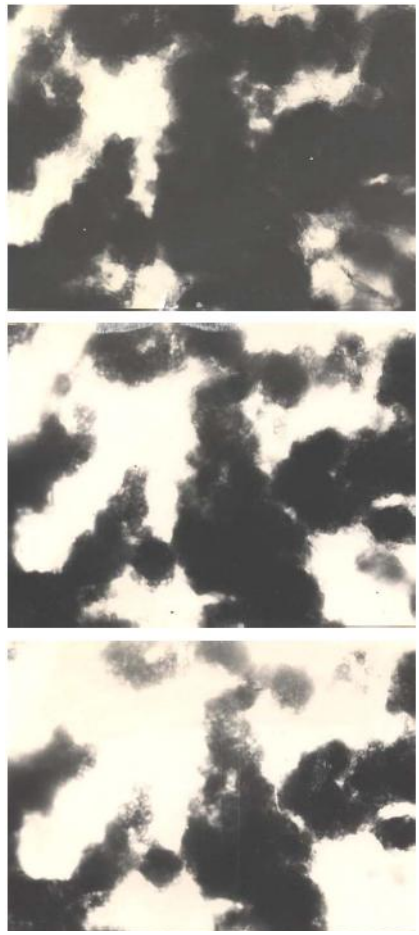



Figure 3. Influence of Verbactin on microorganisms participating in cellulose degradation.

Serozyom soil in autumn is puddle, irrigation and dressing are stopped, soil temperature is lowered, highly developed cotton root system starts dying off, microbial activity runs smoothly. Verbactin expressed prolonged action on bacteria, actynomycetes and fungi, which activated mineralization of plant debris, humic compounds. Actynomycetes and spore bacteria are capable to degrade complex plant materials due to presence of enzymes, and development of cellulose degrading aerobic bacteria and fungi is linked with microbiological schedule and direction of microbiological processes. Quantitative content of actynomycetes – 850, spore bacteria – 250, fungi – 30 thousands CFU/g of soil reveal high mineralization of plant organic matter.

Specific interest was paid to study the Verbactin influence on content of microorganisms-antagonists in cotton rhizosphere. It was established that preparation stimulates growth and development of bacteria, streptomycetes and fungi that inhibit growth and development of Verticillium wilt agent. These cultures induced cotton plants resistance to fungal infection as well. The highest quantity of antagonists was observed in phase of flowering (207 bacterial strains from genera *Bacillus, Pseudomonas, Arthrobacter*, 96 actynomycetes strains from genera *Streptomyces*, *Streptoverticillium*, 70 fungal strains from genera *Aspergillus*, *Penicillium, Fusarium, Stachybotrys, Acremonium, Trichoderma, Gliocladium*). Considerably less number of antagonists was isolated in phase of maturation (91 bacterial strain from genera *Bacillus, Arthrobacter*, 52 actynomycetes from genera *Streptomyces*, *Streptoverticillium*, 40 fungal strains from genera *Aspergillus*, *Stachybotrys, Acremonium, Aspergillus, Penicillium*). The most active antagonists expressing antibiotic activity towards *V. dahliae* (zone of pathogen growth suppression – 20-41 mm) were selected to determine possibility of their use in biocontrol of cotton. It was established that in control plots an increase in number of microorganisms takes place on account of phytopathogens: *Verticillium*

*dahliae*, *V. lateritium*, *Fusarium solani*, *F. oxysporum*, *F. gibbosum*, *Paecilomyces varioti*, *Xanthamonas* sp., *Erwinia sp.*, *Pseudomonas sp.* At the same time test plots were inhabited by fungi-antagonists of *V. dahliae* (*Acremonium*, *Trichoderma*, *Stachybotris*, *Aspergillus*, *Gliocladium*, *Penicillium*).



a – a view of fungus lawn on first days of sclerotia lysis

b – the same spot 2 days later

c – same spot 3 days later

Figure 4. Lysis of *V. dahliae* microsclerotia under action of Verbactin.

Strains possessing growth promoting activity were determined among bacteria, streptomycetes and fungi. These cultures were capable to synthesize phytohormones (indoleacetic and gibberellic acids).

Increased development of phytopathogens in cotton root system suppressed growth and development of cotton and its productivity as well. Thus, healthy plant was 60 cm tall and produced 20 cotton balls, whereas infected plant was 45 cm tall with 16 balls, in average.

According to available data [3], microsclerotia of *V. dahliae* are the main form of its adaptation to preserve, distribute and propagate. That is why study of ability of Verbactin to destroy the infection source in soil – microsclerotia – was conducted.

Lysis of microsclerotia of *V. dahliae* 57 under action of Verbactin was observed at microscope on the same spot (figure 4). The clear gradual lysis of mycelium with microsclerotia was revealed.

The field trials of microbial complex preparation Verbactin reveaed activation of microbiological processes in rhizosphere of cotton plants (variety Navruz) on all stages of its development. At the same time, quantity of useful microorganisms considerably increased compared to control, whereas inhibiting action on phytopathogens including Verticillium wilt agent was observed.

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*Chapter 22*

# FEATURES OF TEACHING OF THE ECOLOGICAL RIGHT AT SAVING BIODIVERSITY OF SOCHI BLACK SEA COAST

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In connection with the great construction of the Olympic objects in Sochi, while training students in the specialization of "Jurisprudence" and studying the discipline of "The Ecological right " there has arisen a sharp necessity to offer additional courses through practical lessons. Within the framework of these course students of the faculty of law attend mandatory training and study ecological problems in concrete conditions of the Olympic construction in Sochi. It will allow further training of highly qualified specialists who will be capable of successfully influencing a complex ecological situation in Sochi while promoting the ecological legislation, and taking into account, the recent infringements in this branch.

By the decision of the Council of ministries' of RSFSR in 1982 the territory in which the Olympic objects are under construction was announced to be an especially protected zone and now is considered to be "The Sochi National park ". In this territory there are rare and disappearing plants and migrational pathways of animals and birds. There are underground water zones that together form as a source for curative waters. The object of the World natural heritage of UNESCO - Caucasian state natural biospheric reserve adjoins to borders the park.

The builders of the Olympic objects in the given territory must observe the Olympic Charter's rules in observance of ecological principles, and also ecological requirements of the International Olympic Committee (IOC) is required. In turn the Government of the Russian Federation expects to result to be a modern resort of the international standard with steady ecology, social and economic development.

However just now ecology of the area of construction is up to the critical level and demands urgent action to be taken. As an example it is possible to show the existence of the city landfill for solid household rubbish. In Adler the landfill is closed, it was maintained for

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more than 60 years, it is planned to open the landfill in Lasarevsky region in the watershed of the rivers Boo and Hobsa, which run into the Black sea and there are mudslides that processes into the channels of the rivers that were already marked (September 8, 2010. AIA news). The realization of immediate actions is needed to protect the nature. The necessity of these actions is written in the Law of Krasnodar Territory March 13, 2008 "№1405" About the legislation of the regional target program "Providing of construction of the Olympic objects and development of Sochi as mountain climatical alpine health resort and balneological resort (2008-2013) ".

"Ecological Vahta on the Northern Caucasus published the materials about illegal cutting down of red - book trees in the territory of the national park- "The Grove of a Pitsunda pine". In the beginning "Trans Yuzh Stroi Company" has destroyed many copies during the construction of a tunnel under Mis Vidniy, and then because of the damage the work was stopped and the construction of an alternative road along the coast of the sea was urgently begun, 50 copies of a Pitsunda pine were cut down and the felling continued.

Imeretinskaya lowland has completely disappeared from an arsenal of the ecologists as a unique place of rest of ancient Colchis and an area of stop for migrational flight of birds. 100 % of Olympic objects are built on this territory. Under the Olympic objects to the depth of 5-8 meters the unique medical mud is buried which the future generations can not use any more.

Understanding the responsibility for the future of the city, the Chairman of the Government of the Russian Federation V.V. Putin has ratified the program of measures on ecological support for the XXII Olympic winter games. The program started to work under the application of Vice-premier D.K. Kozaka (Sochi, September 13, 2010, РИА of News) the situation in the near future will be improved. However, we consider that the improvement of the environment will come with the development of scientific, technical and the educational potential of Sochi, this change is possible at the legislative base in the field of ecology.

The preservation of rare and disappearing plants is very important. Sochi Prichernomorie is the most capacious area of a biological variety in the Russian Federation. For preservation of rare kinds of plants along with others, it is important to use both methods of biotechnology and their juridical support. In this direction the faculty of physiology of the Sochi institute of People's Friendship University works where there are conditions for creation by a method of tissue culture from a bank and accelerate the duplication of certain kinds of plants, which are under the threat of disappearance.

The future lawyers during their practical lessons on the discipline of " the Ecological right " widely study materials performed in mass media, on websites of the ecologists discussed in the ecological projects on TV.

Some research prepared by the students:

"The Order of Government of Russian Federation from 13.11.2010г. № 2017" About independent noncommercial organization "A uniform informational centre";

"WWF: Olympiad in Sochi has already damaged the surrounding nature and countryside";

"The Sochi national park will be cut down in preparation for the Olympic games in 2014";

"Problems of the Black sea and forecasts ";

"Installation of the new modern equipment for ecological monitoring in the resort city of Sochi";

"The Olympics of 2014: some ecological problems and ways to solve them".

The discussion of urgent problems in our lessons promotes a more complete and deeper understanding of the fundamental ecological laws. They understand the necessity for the strict enforcement of the penalties associated with breaking the laws and the importance of ecological control and monitoring. Strict execution of the ecological legislation and the constitutional rights of the citizens is just the beginning.

Only through active interaction of highly qualified specialists will the academic sciences of ecology, economy and jurisprudence ensure the steady development of an ecological sustainable Sochi.

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*Chapter 23*

# CLONAL MICROPROPAGATION ENDEMIC OF THE NORTHERN CAUCASUS MENTHA LONGIFOLIA (L.) HUDS

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## ABSTRACT

We conducted studies to develop methods of introducing the culture *in vitro* micropropagation and grown them in the Black Sea city of Sochi. The valuable plant Mentha longifolia (L.) Huds. Apical stem tips (0,2-0,3 mm) were planted on MS basal medium contained of MS salts, 100 mg $L^{-1}$ myo-inositol, 0.2 mg $L^{-1}$ thiamine HCl, 0.1 mg $L^{-1}$ pyridoxine HCl, 0.2 mg $L^{-1}$ ascorbic acid, 0,5 mg $L^{-1}$ nicotinic acid, 20 g $L^{-1}$ sucrose, and 0.6% cell culture reagent agar. Four to five weeks after initial culture, shoots were selected for our regeneration experiment.

**Keyword**: Mentha longifolia (L.) Huds., Apical stem tips, MS basal medium

Mint – is the genus of aromatic perennial herbs belonging to the family Lamiaceae. These plants are found mainly in temperate and subtemperate areas of the world. Mint has a large number of varieties, which differ widely in characteristics and ploidy level. Several species of genus is considered industrial crops, because they are the sources of essential oils that contain some monoterpenes, widely used in food, aromatherapy industry, cosmetic and pharmaceutical industries. Also mint is an important food crop. It is also worthwhile to consider the use of plants in landscape design. Mint applies to plants exposed to a strong degree of viral infection. It was recently been described a large number of viruses isolated from different varieties of mint grown in the National Clonal Germplasm Repository (NCGR), USA [1]. This review describes the isolated pathogens related to nematode-transmitted, aphid-transmitted, thrips-transmitted, whitefly-transmitted viruses and viruses

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with unknown vectors. This indicates the need for a system improvement of important species of viral pathogens. In the literature, known investigation on improvement of mints from a viral infection by means of meristem culture *in vitro*. The methods for adventitious stem formation [2], direct regeneration from somatic tissues [3]. Tissue culture method has been successfully used to preserve rare varieties [1]. On the Black Sea city of Sochi grow several species of the family Lamiaceae. Including endemic and endangered species Mentha longifolia (L.) Huds. [4]. This is a perennial herb that has a creeping rhizome. Height 0,5 - 1,0 m under favorable conditions can reach 1.5 m high aromatic plant. Leaves paired, opposite, the stem square. Lanceolate leaves 45-100 mm long and 20 mm in width. The leaves are pubescent. Given the economic importance of this species, we conducted a study on the development of a technique of introducing the culture and in vitro micropropagation of this species. Biological material for research was collected near the village of Krasnaya Volya Adler district of Sochi. Selected plants were grown in a greenhouse. We used the young shoots. Nodal segments (0,5-1,0 cm long) were sterilized in a solution of 1.05% sodium hypochlorite with Tween-20 (few drop) for 20 min and then washed with sterile distilled water. Apical stem tips (0,2-0,3 mm) were then placed on medium Van-Hoof [4]. Four to five weeks after initial culture, shoots were selected for our regeneration experiment. These plantlets were cultured in the test tubes containing Murashige and Skoog (MS) basal medium [5] and maintained at 22±2°C and 16 h photoperiod. The MS basal medium contained of MS salts, 100 mg $L^{-1}$ myo-inositol, 0.2 mg $L^{1}$ thiamine HCl, 0.1 mg $L^{-1}$ pyridoxine HCl, 0.2 mg $L^{-1}$ ascorbic acid, 0,5 mg $L^{-1}$ nicotinic acid, 20 g $L^{-1}$ sucrose, and 0.6% cell culture reagent agar. Medium adjusted to pH 5.8 before autoclaving. Received sterile culture (figure 1) are grown in a growth chamber. To develop methods of increasing the multiplication factor and non-sterile rooting cuttings vitro (figure 2). Pretreated in 0.003% solution of NAA micro root cuttings from tubes rooted in vermiculite in plastic

Figure 1. Shoots, regenerated of Mentha longifolia from shoot apical meristem explant with (a) - BAP / NAA (0.2 mg / l / 0.2 mg / l),(b) - BAP / NAA (1.0 mg / l / 0.2 mg / l).

Figure 2. Rooting of microcutting of Mentha longifolia (L.) Huds. in vermiculite.

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*Chapter 24*

# BIOTECHNOLOGY FOR LANDSCAPE CONSTRUCTION IN RUSSIAN SUBTROPICS

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On the occasion of the XXII Olympic Games 2014 Sochi is undergoing extensive reconstruction. Parks, boulevards, and federal and local roads felled for new construction, buildings and private houses knocked down. The less valuable flora as well as unique exotic, rare and endangered plants are died under the ax. A compensation program provides to minimize the damage. However, we can't evaluate the integrity of biodiversity, protected areas and most important thing is we don't know how long it takes the environment to heal such profound changes. So we ought to involve all the latest scientific and technical developments in full use for the conservation and breeding the most valuable of unique plants and attracting to the area of landscape design the local flora's diversity, because the North Caucasus has no equal richness flora diversity throughout the Russian Federation.

It needed to attract a large number of diverse plants to implement the new format of urban greening in the final stages of construction of Olympic venues and infrastructure. For this reason it is necessary to include unique species of native flora, as well as usual species from different regions of the Earth with the same bioclimatic conditions like in our region. The solving of this problem is impossible without modern achievements of biotechnology.

In order to provide training on ornamental gardeners we pay attention to in-depth study of techniques of cell and genetic engineering, which allows multiple increase productivity and provide the introduction of a new generation of cultivars with high levels of compliance with the resource, as well as achieving the maximum reduction of negative environmental impacts. These directions are used in laboratory work on the disciplines of biology. The first year students involve to the scientific work in the biotechnology lab at the Department of Landscape Construction [1] in University of Tourism and Recreation.

Since 2011 the scientific work of students in the department heads to finalize the research paper reflecting the results of scientific work on the in vitro culture of crops recommended as ornamental plants. Students who have received lectures about the latest directions of biotechnology, in particular clonal micropropagation and adventives stem and root formation,

somaclonal variation, haploids, genetic transformation, virus elimination and practical skills in tissue culture in the implementation of scientific research from the first course which completes the head a research paper. Experiments use exclusive highly ornamental plants. In particular studies on micropropagation of prospective plant Eustoma grandiflorum (Raf.) Shinn has been carried out [11,13,14]. In this study, sterile culture were deposited in a bank source material in vitro in the laboratory of Biotechnology used for our traditional breeding and for research on those directions. We took explants from leaves, stems, nodes and roots in experiments on the optimization of the mass proliferation of shoots. This technique is useful for preservation important cultivars in vitro and micropropagation for different purposes. In vitro storage is more convenient than storing in the greenhouse conditions and better than the storage of seeds, because the lines are not completely inbred (homozygous), and the result will not be identical to the parents.

From the analysis of materials literature shows that in Russia, similar studies had not taken place on *Eustoma*. Promising areas of research on the culture were identified in the students works [14]. There were following areas as more promising as model for use in this work with other species:

- Obtaining virus-free plant *Eustoma grandiflorum* (Raf.) Shinn in meristem culture in vitro.
- Adventive regeneration *Eustoma grandiflorum* leaves in sterile culture.
- Getting the somaclonal variants of *Eustoma* from callus.
- Reproduction *Eustoma* by microcuttings.
- Comparative analysis of propagation methods by *Eustoma* cuttings on the physiological and economic characteristics of the plant.
- Study of the conditions of propagation by cuttings of intact plants of *Eustoma*.
- Development project on the use *Eustoma* in the landscape.
- Creation of the collection of *Eustoma in vitro* for year-round production of rooted cuttings.
- The search for new mechanisms of recovery *Eustoma* virus (sterile culture of the stem tops of *Eustoma* together with the tissues of plants producing anti-metabolites *Dianthus cariophyllus* L., *Hypericum perforatum* L. et al).
- Virological assessment of and other plants on Black Sea Coast of Sochi.
- Development of acclimatization techniques ex vitro.
- These directions are developed by students of the Department of Landscape Construction in the University.

In the future, we will broad the range of rare plants for collections in vitro. The most of them are tropical and subtropical ornamental plants which students study in the University on the disciplines like "Southern Ornamental Plants - Dendrology and Floriculture". For the preparation of lectures on this discipline we uses materials of the world's latest researches. Students learn the subject using original papers published in international scientific literature. This discipline is linked very closely with the discipline of "Biotechnology for landscaping in the Subtropical zone".

Sterile plants obtained in the process of research are used in laboratory work with students of the Landscape Construction and Environmental Engineering Faculty of the Sochi

University of Tourism and Recreation in the disciplines "Plant Physiology" and "Biotechnology for landscaping". It is advisable to research results be used to save biodiversity of the Sochi Black Sea coast. The Department of Landscape Construction of the University will provide the direction. Sterile cultures of ornamental plants obtaining in the studies and deposited in a bank of sterile cultures as well as proposed new lines of research can be used for laboratory, projects and dissertations of students of the department and proposed for implementation in the plant industry.

Fundamental knowledge can serve as a basis for applied research: an introduction to sterile culture new species of rare plants of the Caucasus, the identification of new viruses and the development of techniques of their diagnostics, study of new plant growth regulators for in vitro application; establishment of the technologies for eradication of viral infection of the plant tissues; teaching aids; guidelines for the implementation of laboratory work, as well as for preparing and writing a chapter on new methods of mass production of planting material in the research paper; culture sterile plants *Arundo donax* L., *Mentha longifolia* (L.) Huds., *Cyclamen coum* Mill, *Dianthus imerticus* (Rupr.) Schischk., *Colchicum speciosum* Steven, *Epimedium colchicum* (Boiss.) trautv., *Mentha longifolia* (L.) Huds., *Origanum vulgare* L., and other rare plants of the Caucasus.

The results of the experimental work were reported on students' scientific and industrial conferences, 2007-2010 at the University. Results which have been reported are reproduction of roses in vitro [2], the study of plants' virus diseases in the green areas of Sochi [3], the development of regeneration modes in vitro culture of 2 types *Orchidaceae* [4], air conditioning breeding endangered species *Aristolochia steupii* Woronow in vitro [5], micropropagation of *Nephrolepis* [6], The study of growth conditions on *Chrysanthemum* for cut [7], the study of diseased dahlias plants by viruses and develop ways of getting virus-free seed [8], the introduction of the culture in vitro of wild cyclamen [ 9], the study of *Eustoma grandiflorum* reproduction technology [10]. The results were presented at the Moscow International Congress "Biotechnology: the ecology of big cities, 2010, 2011 [11, 12, 17] and in Materials of student scientific conference, SGUTiKD 20-23 May 2010 [13, 14, 15, 16]. As can be seen from the list our work focuses on not only ornamental, but also endangered plants of the Northern Caucasus which should be protected not only from extinction, but to become accessible to the creation of exclusive artificial landscapes, planting of industrial plantations for production of pharmaceutical raw materials, performance export program «Wild Flora from Russia». However, our main aim is the training of highly qualified professionals, possessing the latest developments in biotechnology for the prosperity of our city, and Russia.

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*Chapter 25*

# TASKS FOR TRAINING OF SPECIALISTS FOR BIOTECHNOLOGY IN THE EDUCATIONAL PROCESS IN THE SOCHI BRANCH OF PEOPLE'S FRIENDSHIP UNIVERSITY IN THE AREA OF ENVIRONMENTAL PROBLEMS IN THE REGION

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## ABSTRACT

At the Sochi Institute of People's Friendship University, the opportunities of gaining a deeper understanding of biological disciplines' studies are furthered.

The faculty of Biology was formed at the Department of Physiology. Necessary additions to the educational process will be incorporated according to the new directions among the other professional disciplines which will be the studying of methods of biotechnology and the broadening of opportunities of making new methods to protect endangered plants on a new scale.

**Keywords**: Biotechnology, endangered plants, cell culture

At the Sochi Institute of People's Friendship University teachers use modern methods of training specialists and these methods let students carry out in-depth research of the foundations of biotechnology by using biotechnology and molecular biology. The institute of Bioorganic Chemistry named M.M Shemyakin and Yu.A. Ovchinnikov RAN signed a contract which gives the senior students of the Department of Physiology the opportunity to fulfill their research and thesis work at the Scientific-Educational Center of Research and

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development. Additional Education Institute held a joint training session, “School for Young Scientists” in Sochi on the basis of the Sochi Institute of People's Friendship University.

A prerequisite for the development of cooperation with the Institute of Bioorganic Chemistry is a program where students master modern methods of cell and genetic engineering. With the development of these methods endangered and rare Plants are used as the biological material. These plants are valuable as objects of ecological conservation programs help aid in the recovery of endangered habitats and landscapes of flora which are found in the wild.

The studying of this method helps deepen the understanding of cell engineering [1].

Currently we are expanding the organizational structure of the Department: preparing the transformation of the biological faculty, which includes the Department of Biology and Ecology and the Department of Veterinary Medicine. Along with other relevant disciplines there will be different opportunities for the furthering of biological knowledge. Important direction in improving of qualified characteristics of the future specialists is taking part in different kinds of scientific conferences. So, in 2010, students participated in the poster session at the Moscow International Congress "Biotechnology: the ecology of large Cities. The report presents the material using methods of cell engineering in the restoration of endangered species [2].

In 2009-2010 the Sochi branch of People's Friendship University was audited by the Commission of the Ministry of Education, which aimed to improve the quality of education and the level of methodological support of educational programs, implementation of priority directions of science and technology.

The result of the Commission was that the Sochi's branch of People's Friendship University was upgraded to the Institute of People's Friendship University. This has enabled the Institute to continue working on and improving the quality of education. Incorporating the work of the department of physiology with the use of biotechnology in the teaching of natural sciences, students of the biological faculty will take part in the work of this Biospherical Reserve and these areas with the status of “specially protected zones”. With the rapid Olympic construction, limited target dates for commissioning of facilities, it is very difficult to monitor compliance with environmental sustainable development of the Sochi region. During construction, experts identified a number of violations. Experts from the (IOC) International Olympic Committee and our ecologists say that the effects of the inevitable interference with the unique nature of the Sochi Black Sea during preparation for the Olympics are so great that these pristine spaces have been so damaged that it will take many years to heal. Understanding this, it is clear that well trained ecologists will be in great demand. Researching in the selection of medicinal plants during the rehabilitation of athletes and tourists there will be a fundamentally new direction in the work of the faculty.

Another direction of work associated with of the Faculty of Biology is connected with the problems of comprehensive examination and rehabilitation of athletes especially at the professional level.

It is necessary to prepare experts who are participating in the process of preparation of athletes, skillfully guiding them during the competition, monitoring their health after finishing the competitions and supervising rehabilitation.

The new status of the institute will enhance graduate education. As preparations are underway for the opening of the Science Education Center (REC), the center will focus on conducting research and training specialists in medical and other fields. Leading scientific

and educational institutions in Sochi: Sochi Institute of People's Friendship University, Institute of Medical Primatology, RAMS, Institute of Floriculture and Subtropical Crops, as well as Medical University and Institute of Medical Primatology, Abkhazia take part in the formation of the Scientific Educational Center.

In the structure of this center (SEC) it will be possible to improve the level of the specialists which are in short demand in this region. The research of rare and endangered plants will be furthered in the area of Sochi Black Sea Coast which is the most capacious enclave in all of Russia. Here, methods are needed for biotechnology. Cellular engineering in combination with modern methods of plant virology is an effective basis for the introduction of these plants in conservation of them, creating the possibility of using these valuable plant-based methods for the new generation.

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