Ramesh Chander Kuhad
Ajay Singh *Editors*

Biotechnology for Environmental Management and Resource Recovery

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Foreword

It gives me immense happiness to write the foreword for the excellent compendium *Biotechnology for Environmental Management and Resource Recovery* edited by Prof. Ramesh Chander Kuhad and Dr. Ajay Singh. The constant increase in world population has put pressure on urban development and agricultural productivity. Both have led to the generation of huge quantity of wastes (solid and liquid). Inappropriate management of liquid and solid waste not only increases the pollution to the environment but also threatens human health. Therefore, the sustainable waste management technologies are necessary to keep the environment clean and green.

In my opinion, solid waste disposal has comparatively bigger challenges than liquid waste management. There are consistent efforts by researchers to continuously develop new technologies for utilizing the solid wastes, especially the plant biomass which represents a major part of the available solid wastes and represents a renewable source of energy and chemicals. Various microbial processes have been developed for bioconversion of agro-residues and grasses into bioethanol as alternative fuel, digestible animal feed, and value-added chemicals.

I am delighted that an edited book on "Biotechnology for Environmental Management and Resource Recovery" is being brought out, which discusses a wide range of selected topics related to the applied and fundamental aspects of potential utilization of agro-residues into value-added products and processes and applications of microbial technologies for bioremediation and monitoring of environmental pollutants. Chapters dealing with industrial applications address current biotechnological approaches based on microbial enzymes such as cellulases, ligninases, pectinases, and phytases.

This book has contributions from academicians and scientists from different disciplines including microbiology, molecular biology, genetics, and environmental biotechnology with diverse backgrounds and from universities, national laboratories and institutes, and industries. I am confident that the book should prove to be very useful to the students, researchers, scientists, and teachers of microbiology, environmental science and engineering, and biotechnology.

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Preface

The constant increase in urbanization and industrialization around the world has immensely affected the environment. The textile, pulp and paper, petrochemicals, minerals, energy, food and feed, and other agro-based industries produce huge amount of waste, which enters in terrestrial and aquatic environments and eventually leads to environmental disturbances. Various types of agricultural, forestry, and fruit and vegetable processing wastes represent important energy and material resources and therefore require proper management to avoid the contamination of the environment and spoilage of valuable natural resources.

In the absence of inadequate waste management strategies, especially, the developing countries suffer from severe environmental problems and shortage of energy and resources and food production. Thus, there is a need to have simple and economically viable and integrated technologies for environmental management and resource recovery. Microorganisms and their enzymes contribute significantly to residual biomass utilization, resource recovery through recycling and conservation, and environmental management. Recently, advances in microbiological, molecular, and enzymatic processes have motivated in-depth studies to exploit microorganisms and their enzymes for developing innovative biotechnologies.

Biotechnology for Environmental Management and Resource Recovery presents a comprehensive review of selected research topics related to environmental pollution management and developing biotechnologies based on bioconversion of agro-residues (lignocellulosics) into biofuels, animal feed, paper, etc. Moreover, rhizobacteria-based technology in agroecosystem management has been discussed. The book comprises 16 chapters, divided in two parts. Part I includes topics on microorganisms and enzymes involved in lignin degradation vis-à-vis production of nutritionally rich animal feed, microbial cellulases, pectinases, enzymatic retting, biofuels, rhizobacteria and agricultural management, and sustainable enzyme technology for resource recovery. Part II includes the aspects of applied biocatalysis, metagenomics and genetically modified microorganisms (GMOs), and microbial enzymes in environmental management and bioremediation.

The book contains contributions from leading scientists with diverse backgrounds of academic institutions and industries. This book will be of immense use to the students of environmental biotechnology, microbiology, biochemistry, and other environmental sciences and engineering. This book will

certainly be of equal interest to the teachers, scientists, and researchers, whether in academia, industry, or government, directly involved in the research or who want to learn about environmental management and the efficient utilization of natural resources via biotechnological applications.

Editors

Ramesh Chander Kuhad
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About the Book

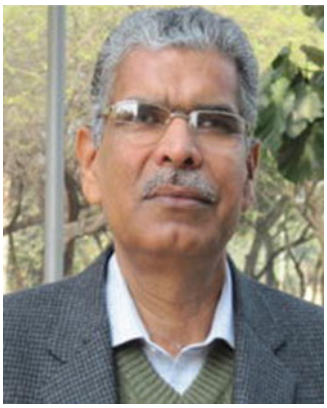
Various types of secondary agricultural and forestry wastes represent valuable resource materials for developing alternate energy as biofuels and other value-added products such as sugars, phenols, furans, organic acids, enzymes, and digestible animal feed. However, if not managed properly, waste material and environmental contaminants generated by various industries such as food and feed, pulp and paper, and textile may lead to severe environmental pollution. The energy, food, and feed demand necessitate developing simple and economically viable technologies for environmental management and resource recovery. Microorganisms and their enzymes contribute significantly in the utilization of plant residues, resource recovery, and eventually in pollution mitigation.

Biotechnology for Environmental Management and Resource Recovery presents a comprehensive review of selected research topics in a compendium of 16 chapters related to environmental pollution control and developing biotechnologies in agro-ecosystem management and bioconversion of agro-residues (lignocellulosics) into biofuels, animal feed, paper, etc. This book provides a valuable resource for reference and text material to graduate and postgraduate students, researchers, scientists working in the area of microbiology, biotechnology, and environmental science and engineering.

Editors

Ramesh Chander. Kuhad
Ajay Singh

About the Editors



Dr. Ramesh Chander Kuhad, M.Sc., M.Phil, and Ph.D. (microbiology), is currently a Professor of Microbiology in the Department of Microbiology and Joint Director, Institute of Lifelong Learning, University of Delhi South Campus, New Delhi, India. The main focus of his research is on microbiology and biotechnology of lignocellulose bioconversion. Over all, the main theme of his research is to make use of waste lignocellulosics and the crop by-products in developing value-added products. He has published more than 120 peer-reviewed research papers and chapters with SCI more than 1,500 (since 1996) and three books. Dr. Kuhad is a member of executive council/ planning and monitoring board, academic council, and governing bodies of various educational institutes. He has also served as Treasurer (9 years), General Secretary (6 years) and President of Association of Microbiologists of India (AMI). He has been the recipient of commonwealth scholarship, UNIDO-ICGEB Short-Term Fellowship, Long-Term Overseas Research Associateship, and Short-Term Biotechnology Overseas Associateship awards.

Dr. Ajay Singh is the Technical Director of Lystek International Inc., Cambridge, and Adjunct Faculty Member at the University of Waterloo, Ontario, Canada. He has authored/edited ten books in the areas of biotechnology and applied bioremediation and published around 200 peer-reviewed research papers and book chapters; He holds 12 international patents. Dr. Singh has 25 years of experience in industrial research/process development and designing various bioreactor-based processes related to wastewater, bioremediation, fermentation, and food industries. He is currently an advisor to various international environmental companies and academic organizations in Canada, USA, Asia, and Middle East. He has been honored with a young scientist award by Association of Microbiologists of India and is currently a Fellow of National Academy of Biological Sciences and National Academy of Agricultural Sciences of India and is an active member of international societies such as Water Environment Association of Ontario and Water Environment Federation.

Part I

1 Microorganisms and Enzymes Involved in Lignin Degradation Vis-à-vis Production of Nutritionally Rich Animal Feed: An Overview

Ramesh Chander Kuhad, Sarika Kuhar,
Krishna Kant Sharma, and Bhuvnesh Shrivastava

Abstract

Lignocellulosics are the major structural component of woody and nonwoody plants and represent a major source of renewable organic matter. The plant cell wall consists of three major polymers: cellulose, hemicellulose, and lignin. Lignocellulose biomass, available in huge quantity, has attracted considerable attention as an alternate resource for pulp and paper, fuel alcohol, chemicals, and protein for food and feed using microbial bioconversion processes. The current industrial activity of lignocellulosic fermentation is limited because of the difficulty in economic bioconversion of these materials to value-added products. Lignin is degraded to different extents by variety of microorganisms including bacteria, actinomycetes, and fungi, of which wood-rotting fungi are the most effective, white-rot fungi in particular. White-rot fungi degrade wood by a simultaneous attack on the lignin, cellulose, and hemicellulose, but few of them are specific lignin degraders. The selective lignin degraders hold a potential role in economically bioconversion of plant residues into cellulose-rich materials for subsequent bioethanol and animal feed production. Different fungi adapt in accordance to conditions existing in the ecosystem and complete their task of carbon recycling of the lignified tissues, and some white-rot fungi have capability to completely mineralize it. It is known that white-rot fungi are able to perform lignin degradation by an array of extracellular oxidative enzymes, the best characterized of which are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. However, the regulation of the production of individual enzymes and lignin degradation is a complex phenomenon.

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Unfortunately, even selected white-rot fungi take long in delignifying the lignocellulosic substrates. Therefore, it is necessary to improve these fungi for their ability to degrade lignin through various conventional and modern approaches. A considerable progress has been made in this direction during the past two decades; LiP, MnP, and laccase genes have been cloned, and an efficient *Agrobacterium*-mediated transformation system has been developed, which will eventually help in successful expression of the desired protein. This chapter presents an overview of diversity of lignin-degrading microorganisms and their enzymes especially in developing animal feed. In addition to that, advances in molecular approaches to enhance the delignification capability of microorganisms are also discussed.

Introduction

The residues from agricultural crops and agro- and forest-based industrial processes are abundantly available worldwide. Most of these agricultural crops and forest by-products are deficient in protein content, and presence of lignin in cell wall limits the digestibility of these materials in ruminants. Therefore, attempts have been made around the world to improve their protein content and digestibility by fermenting them under solid-state fermentation (SSF) conditions, using various microorganisms. Among these, white-rot fungi are considered the most capable of successfully degrading lignin. Most of them are slow colonizers and degrade cellulose and hemicellulose along with the lignin and are called as simultaneous degraders. However, recently a few white rots have been reported, which degrade lignin selectively (Akhtar et al. 1997; Hakala et al. 2004, 2005; Okano et al. 2009; Gupta et al. 2011; Shrivastava et al. 2011). Most of the work on solid-state fermentations (SSF) of agroresidues have been carried out using simultaneous degrader, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*, the imperfect form), the most studied lignin-degrading fungus (Eriksson et al. 1990; Kuhad et al. 1997; Basu et al. 2002; Kumar et al. 2006; Gassara et al. 2010). However, recently selective lignin-degrading fungi such as *Lentinus edodes*, *Pycnoporus cinnabarinus*, *Crinipellis* sp. RCK-1, *Ceriporiopsis subvermispora,* and *Phlebia brevispora* are being studied for lignin degradation (Okano et al. 2006, 2009; Kuhar et al. 2008; Arora et al. 2011).

Some white-rot fungi are capable of degrading lignin selectively but the enzymes produced by them are too low for commercial purposes. To improve the enzyme production by selective white-rot fungi, various attempts have been made to optimize the culture conditions, and considerable increase in production of laccase, an important lignin-degrading enzyme, has been achieved (Dhawan et al. 2004; Sharma et al. 2005; Bonugli-Santos et al. 2010). For a commercial perspective, there is need to optimize SSF at pilot scale using selective fungi. The ligninolytic degradation ability of the selective degraders can be improved by using traditional mutagenesis and modern molecular techniques like recombinant DNA technology, and there has been a significant progress in this direction (Sharma and Kuhad 2010).

Genome sequence of the well-known lignin-degrading fungus, *P. chrysosporium,* has been unraveled (Martinez et al. 2004). This fungus possesses an impressive array of genes encoding extracellular oxidative enzymes: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Ten LiP genes and five MnP genes have been found. Genome searches revealed no conventional laccases. Instead, four multicopper oxidase (MCO) sequences are found clustered

within a 25-kb segment on scaffold 56. Thus, it appears that *P. chrysosporium* does not have the capacity to produce laccase although distantly related multicopper oxidases may have a role in extracellular oxidation. The genome harbors the genetic information to encode more than 240 putative carbohydrate-active enzymes (http://afmb.cnrs-mrs.fr/CAZY/) including 166 glycoside hydrolases, 14 carbohydrate esterases, and 57 glycosyltransferases, comprising at least 69 distinct families.

A number of genes encoding fungal laccases, lac 1, lac 2, lac 3, lac 4, and lac 5 and other important lignin-degrading enzyme(s), have been cloned including those from basidiomycetous fungi such as *Trametes (Coriolus) versicolor, T. villosa, Coriolus hirsutus*, *Rhizoctonia solani, Agaricus bisporus, Phlebia radiata,* Basidiomycete PMI, *P. cinnabarinus, and C. subvermispora* and ascomycetous fungi such as *Cryphonectria parasitica, Aspergillus nidulans, Podospora anserina,* and *Neurospora crassa* (Leonowicz et al. 1999). Recently, cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea* is carried out (Huang et al. 2009).

The ligninolytic enzymes are difficult to express in non-fungal systems. Laccase has been reported to be expressed in *Saccharomyces cerevisiae, Trichoderma reesei, and Aspergillus oryzae*. However, to the best of our knowledge, reports about purification and characterization of the recombinant proteins are scanty. The expression level has been low in most of the systems tested so far. The overexpression using specific vectors and promoters may provide higher yields.

Here we will discuss in detail about the diversity of lignin-degrading microorganisms, their enzymes, applications, and future prospects in developing biotechnological approaches, especially in developing animal feed.

Composition of Lignocellulosic Materials

Lignocellulosics in the form of wood and agricultural residues are virtually inexhaustible, since their production is based on the photosynthetic processes. They are the most abundant renewable natural material in the biosphere, accounting for approximately 50% of the total biomass present in the world ($10–50 \times 10^9$ t) (Sun and Cheng 2002; Nair 2006; Sánchez 2009). Regardless of source, lignocellulosic material contains three types of polymers – i.e., cellulose, hemicellulose, and lignin – that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. Cellulose is polymer of glucose with cellobiose as repeating units, and hemicelluloses are macromolecules from different sugars, whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions. Table 1.1 shows the typical compositions of these three components in hardwoods, softwoods, agricultural residues, and various other lignocellulosic materials.

Lignin-Degrading Microorganism and Their Enzymes

A wide range of microorganisms such as bacteria, actinomycetes, cyanobacteria, and fungi are known for degradation of lignin; however, the level of degradation varies with microorganisms. The degradation and transformation of lignocellulosic wastes is attributed to the metabolism of indigenous microorganisms. Different microbial population dominates at various stages and has distinct roles in degradation of organic matter (Belyaeva and Haynes 2009).

Lignin-Degrading Bacteria

The degradation of wood cell wall by bacteria was not ascertained until the 1980s (Li et al. 2009). Bacteria generally degrade wood slowly, and degradation takes place on wood surfaces with high moisture content. Because of lack of penetrating ability, bacteria usually invade wood cells simultaneously with fungi. They appear to attack both softwoods and hardwoods by first colonizing the

Table 1.1 The contents of cellulose, hemicellulose, and lignin in some wood and common agricultural residues and wastes

	% dry weight		
Lignocellulosic materials	Cellulose	Hemicellulose	Lignin
Hardwoods stems			
Aspen	50.0	28.0	15.0
Beech	47.0	20.0	23.0
Birch	41.0	26.0	25.0
Cottonwood	46.0	19.0	24.0
Oak	48.0	18.0	28.0
Poplar	45.0	19.0	20.0
Red Maple	39.0	33.0	23.0
Softwood stems			
Douglas fir	57.0	8.0	24.0
Eastern hemlock	43.0	10.0	32.0
Jack pine	41.0	10.0	27.0
White pine	44.0	11.0	28.0
Red spruce	43.0	12.0	27.0
White spruce	44.0	10.0	27.0
Agricultural residues			
Bagasse	33.0	30.0	29.0
Barley straw	40.0	20.0	15.0
Corn cob	42.0	39.0	14.0
Cotton stalks	42.0	12.0	15.0
Groundnut shells	38.0	36.0	16.0
Oat straw	41.0	16.0	11.0
Rice straw	32.0	24.0	13.0
Rye straw	37.0	30.0	19.0
Wheat straw	30.0	24.0	18.0
Cotton seed hairs	80–95	5.0–20.0	0
Others			
Grasses	25.0–40.0	35.0–50.0	10.0–30.0
Paper	85.0–99.0	0.0	0–15.0
Sorted refuse	60.0	20.0	20.0
Leaves	15.0–20.0	80.0–85.0	0
Newspaper	40.0–55.0	25.0–40.0	18.0–30.0
Waste papers from chemical pulps	60.0–70.0	10.0–20.0	5.0–10.0
Primary wastewater solids	8.0–15.0	NA	24.0–29.0
Swine waste	6.0	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25.0	35.7	6.4
Switch grass	45.0	31.4	12.0

parenchyma cells. After utilizing the cell contents, they may also attack the parenchyma cell walls (Liese 1970). They move into adjacent cells and tracheids with fast disruption of pits (Levy 1975). Different patterns of cell wall decay, cavitation, and tunneling by bacteria have been found both in natural and laboratory environments (Nilsson and Daniel 1983; Daniel et al. 1987).

In contrast, studies in some laboratories have shown that bacteria are unable to degrade lignified

plant cell walls (Schmidt et al. 1987), but they were able to do so after chemical pretreatment of the cells. Efficient bacterial degradation of wood already treated with cellulase-less mutants of *P. chrysosporium* and *Phlebia gigantea* has been observed.

Although bacteria can directly attack fibers, vessels, and tracheids, few species or strains can degrade all the cell wall components (Eriksson et al. 1990). However, some bacteria have been found to degrade lignified wood cells, which were confirmed by ultrastructural investigations (Zimmermann 1990). Recently, cell wall erosion (Sutherland et al. 1979), tunnel formation (Daniel et al. 1987), and removal of lignin by bacteria have also been reported (Krause et al. 2003). Recently, *Paenibacillus* sp. (AY952466), *Aneurinibacillus aneurinilyticus* (AY856831), and *Bacillus* sp. (AY952465) for ITRC S6, IITRC S7, and ITRC S8, respectively, were found capable to effectively degrade the kraft lignin, a major by-product of the chemical pulping process and main contributor to the color and toxicity of effluent (Chandra et al. 2007).

The enzyme produced by bacteria can catalyze the cleavage of interunit linkage of model lignin dimers (Archana and Mahadevan 2002; Li et al. 2009). The contribution of bacteria to the complete biodegradation of lignin in natural environment where fungi are also present is not much known. However, bacteria seem to play a leading role in decomposing lignin in aquatic ecosystem, because wood-degrading bacteria have a wider tolerance of temperature, pH, and oxygen limitations than fungi (Chandra et al. 2007). Molecular evidence for occurrence of lignin-degrading enzymes has been found in *Mycobacterium tuberculosum*, *M. avium, Escherichia coli*, *Caulobacter crescentus*, *Pseudomonas syringae*, *P. aeruginosa, P. putida, Bordetella pertussis*, *Xanthomonas campestris*, *Rhodobacter capsulatus*, *Yersinia pestis*, *Campylobacter jejuni,* and *Aquifex aeolicus* (Alexandre and Zhulin 2000).

Hungate (1966) discussed early examples in which fibrolytic bacteria dosed in to rumen had little effect on rumen digestion. Rumen bacteria are major degraders of plant fiber cell walls by production of enzymes active against structural components of these cell walls (Akin 1993a; Kuhad et al. 1997; Krause et al. 2003). Some of the most extensively studied rumen bacteria include *Fibrobacter succinogenes, Ruminococcus albus,* and *R. flavefaciens*. These bacteria have a complete set of polysaccharide-degrading enzymes and also the ability to adhere to fibers (Stewart and Bryant 1988; Akin 1993a). These species adhere strongly to partially degraded cell walls but erode the components only if they are adjacent and in direct contact with the bacteria (Krause et al. 2003). Often, the plant cell walls are totally degraded, but at other times digestion seems to be interrupted before the hydrolysis is completed. Very recently the emerging role for bacteria in lignin degradation and bio-product formation has been reviewed and elaborated (Bugg et al. 2011). A range of soil bacteria, often aromatic-degrading bacteria, are able to break down lignin. The enzymology of bacterial lignin breakdown is currently not well understood, but extracellular peroxidase and laccase enzymes appear to be involved. There are also reports of aromatic-degrading bacteria isolated from termite guts, though there are conflicting reports on the ability of termite gut microorganisms to break down lignin.

In brief, bacterial strains identified to have activity for lignin breakdown fall into three classes: actinomycetes, α-proteobacteria, and γ-proteobacteria (Bugg et al. 2011). The activity of these lignin-degrading bacteria was much less than that of white-rot fungus *Phanerochaete chrysosporium* but was found comparable to some other lignin-degrading fungi. *P. putida* mt-2 and *R. jostii* RHA1 were found to break down lignocellulose, releasing low molecular weight phenolic products (Ahmad et al. 2010).

The possible involvement of bacteria in lignocellulose breakdown has also emerged from a different line of research. The gut microflora of termites helps to digest the lignocellulose content of wood, but the role of microorganisms in the degradation of lignin has been the subject of debate (Brune 2007). A recent study by Geib et al. (2008) has shown that depolymerization, demethylation, and ring hydroxylation of lignin

occur using gut microflora from *Anoplophora glabripennis* and *Zootermopsis angusticollis* and have suggested that the aerobic reactions required for lignin depolymerization could occur in the foregut, rather than in the largely anaerobic hindgut. The enzymology for bacterial lignin degradation is at present poorly understood, compared with the fungal lignin-degrading enzymes, yet there are indications that bacteria use similar types of extracellular lignin-degrading enzymes to fungi. Lignin-degrading *S. viridosporus* T7A produces several extracellular peroxidases, which have been shown to catalyze oxidative cleavage of β-aryl ether lignin model compounds (Ramachandra et al. 1988). There are also reports of bacterial laccases, which are copper-containing enzymes that utilize oxygen to oxidize a range of phenolic compounds. While the bulky nature and presence of nonphenolic subunits prohibits the action of laccases on the lignin polymer, they have been shown to depolymerize lignin via the oxidation of smaller molecules such as 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HBT) (Ten Have and Teunissen 2001; Sánchez 2009). In addition to their role in the degradation of lignin, the broad substrate specificity of laccases permits the modification of lignocelluloses and the potential to create new, environmentally friendly materials. The potential of bacteria in bioconversion of lignocellulosics may further be explored in the future.

Lignin-Degrading Actinomycetes

Actinomycetes are a group of eubacterial microorganisms, also called as actinobacteria, that are commonly found in the soil. The presence of actinomycetes in the soil is indisputable; what is not clear is their role in the ecology of the soil. It has been suggested that actinomycetes play a role in lignocellulose breakdown, but details of the scale and methods by which such breakdown takes place are less known. However, there is a wide range of examples where *Streptomyces* and other actinomycetes have been identified as degrading lignin or lignocellulose. These strains come from a wide variety of sources, including a range of soils, high temperature environments, and termite guts (Adhi et al. 1989; Iqbal et al. 1994; Ruttiman et al. 1998; Tuncer and Ball 2002; Watanabe et al. 2003). Different species of *Streptomyces* have been reported to colonize vessels, fibers, and parenchyma cells. *S. flavovirens* rapidly colonizes the phloem and degrades parenchyma cells as well as thick-walled highly lignified sclereids. In advanced stages of degradation, parenchyma cells were found to be completely destroyed, and sclereids showed evidence of eroded cell walls. Various studies have established that several actinomycetes attack grass lignocellulosics, leading to the partial solubilization of the substrate rather than mineralization (Kuhad et al. 1997).

A wide range of actinomycetes have been shown to possess extracellular peroxidase activity, though the activity was variable (Mercer et al. 1996). Laccase type enzymes have now been found in five actinomycetes: *Streptomyces antibioticus* (Freeman et al. 1993), *S. griseus, S. coelicolor* (Endo et al. 2003a, b), *S. cyaneus* (Arias et al. 2003), and *S. lavendulae* (Suzuki et al. 2003). However, it is not clear how widespread they are in the actinomycetes because there is indirect evidence for the presence of laccases in the actinomycetes, which is based on rather nonspecific substrate reactions (Sjoblad and Bollag 1981). Thus, with the availability of 17 complete genome sequences and six partially completed genome sequences from the actinobacteria, the possibility of identifying candidate laccases from the actinomycetes is possible using a bioinformatics-based approach. However, these sequenced genomes include only one thermophile, *Thermobifida fusca,* which has not been completely annotated (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi; last accessed 4 January 2005).

Peroxidase and polyphenol oxidase activity have been compared between the thermophilic *Streptomyces* isolates and mesophilic *Streptomyces* (Mhlanga 2001). Polyphenol oxidase activity between the two groups was comparable, and peroxidase activity was significantly higher for the thermophiles than for the mesophiles.

Table 1.2 Types of wood-rotting fungi and some characteristics

Type	Family to which wood rot belongs	Texture of wood after attack by wood rot	Components of wood degraded
Soft rot	Ascomycetes and Deuteromycetes	Wood remains wet, spongy, pitted, and brownish. Usually grows on surface of wood and is least specialized among wood rots	Prefer carbohydrates and modify lignins to a limited extent
Brown rot	Basidiomycetes	Wood remains fibrous and brownish	Primarily carbohydrates degraded, leaving behind brownish modified lignin but does not degrade it
White rot	Basidiomycetes and Ascomycetes	Wood remains fibrous and whitish as cellulose mainly is left behind	All components of wood degraded

RU-A01, RU-A03, and RU-A06 also have an advantage over the mesophilic *Streptomyces* strains because they produce lignin peroxidases also found among some of the white-rot fungi and are known to play a role in lignin solubilization (Ball et al. 1989). Peroxidase and polyphenol oxidase extracts from the thermophilic isolates react the same way as the peroxidases and polyphenol oxidases from the mesophilic *Streptomyces* sp. toward the various substrates. In a recent study Huang et al. (2010) demonstrated the change in microbial population while degrading lignin during composting process through quinine profiling and concluded that high lignin degradation at cooling stage might be attributed to the cooperation of mesophilic fungi, actinomycetes, and bacteria.

Lignin-Degrading Fungi

Wood-Rotting Fungi

By colonizing dead or dying tree trunks and stumps, the fungus preferentially utilizes one or the other cell wall constituents, resulting in the wood decay known as wood rot. These include three types of wood rots, i.e., soft rot, brown rot, and white rot, which are based on the component utilized and the color characteristics of the decayed wood (Table 1.2). Among them, only white rots have the potential to completely degrade all three major components of wood, thus making them ecologically most important to study their detailed diversity. These fungi mainly belong to Ascomycetes, Deuteromycetes, or Basidiomycetes group (Dashtban et al. 2009).

Table 1.2 represents diversity of wood-decaying fungi and their ligninolytic system. Soft rot results in degradation of cellulose and hemicellulose, but lignin may only be partially digested. Little is known about the degradation mechanism of lignocellulose by soft-rot fungi in contrast to white- and brown-rot fungi. However, it is clear that some soft-rot fungi can degrade lignin because they erode the secondary cell wall and decreases the content of acid-soluble material (Klason) in angiosperm wood (Sánchez 2009). Ascomycetes and Deuteromycetes (fungi imperfecti) generally cause soft-rot decay of wood (Blanchette 1995). Two forms of soft rot have been described, type I consisting of biconical or cylindrical cavities that are formed within secondary walls while type II refers to an erosion form of degradation (Blanchette 1995).

In contrast to nonselective white-rot fungi, the middle lamella is not attacked by type II soft-rot fungi. Xylariaceous ascomycetes from genera such as Daldinia, Hypoxylon, and Xylaria have earlier often been regarded as white-rot fungi, but nowadays, these fungi are grouped in soft-rot fungi since they cause typical type II soft rot. Ligninolytic peroxidases or laccases of soft-rot fungi may not have the oxidative potential to attack the recalcitrant guaiacyl lignin. On the other hand, syringyl lignin apparently is readily oxidized and mineralized by the enzymes of soft-rot fungi (Nilsson et al. 1989). Soft-rot fungi are particularly prevalent at the early stages of wood

decay and in conditions of high moisture and increased nitrogen content (Blanchette 1995). Wood affected by soft rot may appear wet, spongy, or pitted. There are over 300 species of known soft rots (Kuhad and Singh 1993). Soft rots are either the members of Ascomycetes or Deuteromycetes (Kuhad et al. 1997) or some genera falling under this category are *Chaetomium*, *Paecilomyces,* and *Fusarium* (Rayner and Boddy 1988; Eriksson et al. 1990; Blanchette 1995). Ascomycetes are known to degrade lignin and are responsible for wood decay referred to as "soft rot," a process that is not well understood (Shary et al. 2007).

The fungi capable of degrading cellulose and hemicellulose but are unable to digest the lignin component of wood belong to brown-rot category (Sánchez 2009). In this case, the lignin remains more or less intact and becomes modified with brown and crumbly matrix appearance (Eriksson et al. 1990; Blanchette 1995; Highley and Dashek 1998). Brown-rot fungi mainly decompose the cellulose and hemicellulose components in wood (Eriksson et al. 1990). They have been much less investigated than white-rot fungi in spite of their enormous economic importance in the destruction of wood. Brown-rotted wood is dark, shrinks, and is typically broken into brick-shaped or cubical fragments that easily break down into brown powder (Blanchette 1995). The brown color indicates the presence of modified lignin in wood. Unlike the soft rots, the brown-rot fungi are relatively few in number, comprising only 6% of all wood-rotting Basidiomycetes (Gilbertson 1980; Rayner and Boddy 1988; Kuhad et al. 1997).

Brown-rot fungi produce low molecular weight chelators which are able to penetrate in to the cell wall (Dashtban et al. 2009). *Poria* sp., *Polyporus* sp., *Coprinus* sp., *Gloeophyllum trabeum, Laetiporus sulfurous, Wolfiporia cocos, Piptoporus betulinus,* and *Fomitopsis* sp. are some examples of brown-rot fungi (Buswell and Odier 1987; Rayner and Boddy 1988; Eriksson et al. 1990; Straatsma et al. 1994; Blanchette 1995; Dix and Webster 1995; Machuca and Ferraz 2001; Valaskova and Baldrian 2006; Dashtban et al. 2009; Deswal et al. 2011). The fungal hyphae penetrate from one cell to another through existing pores in wood cell walls early in the decay process. The penetration starts from the cell lumen where the hyphae are in close connection with the S3 layer. In brown rot, the decay process is thought to affect the S2 layer of the wood cell wall first (Eriksson et al. 1990). Brown-rot fungi have a unique mechanism to break down wood polysaccharides. In contrast to white-rot fungi that successively depolymerize cell wall carbohydrates only to the extent that they utilize hydrolysis products in fungal metabolism, brown-rot fungi rapidly depolymerize cellulose and hemicellulose, and degradation products accumulate, since the fungus does not use all the products in the metabolism (Cowling 1961). To some extent, brown-rot fungi have similar degradative capabilities and pathways as white-rot fungi. Both wood-decay mechanisms rely on radical formation, low pH, and the production of organic acids. They cause increased alkali solubility of lignin, and the decay is enhanced by high oxygen tension, all of which indicate a crucial involvement of radicals, especially in the early stages of decay.

White-rot fungi are capable of degrading all the major components of wood (cellulose, hemicellulose, and lignin) (Sánchez 2009). The fungi causing white rots mostly belong to Basidiomycetes (Table 1.3), but some Ascomycetous fungi have been also identified to result wood decay (Eriksson et al. 1990) such as *Xylaria hypoxylon* and *X. polymorpha* commonly known as candle snuff fungus and dead man's finger, respectively, (Deacon 1997); *Ustulina vulgaris* (Kuhad et al. 1997) have been reported to aid in lignin degradation.

White-rot fungi are a heterogeneous group of fungi classified in the Basidiomycota. Different white-rot fungi vary considerably in the relative rates at which they attack lignin and carbohydrates in woody tissues. Some remove lignin more readily than carbohydrates, relative to the original amount of each (Blanchette 1995). Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones increase as decay progresses, and large voids filled with mycelium are formed. This type of rot is referred to as

Table 1.3 Diversity of wood-decay fungi and their ligninolytic enzymes

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
1.	*Abortiporus biennis*	Meripilaceae	MnP/Lac	Aggelis et al. (2002)
2.	*Agaricus bisporus*	Agaricaceae	MnP/Lac	Bonnen et al. (1994)
3.	*Agrocybe praecox*	Bolbitiaceae	MnP/Lac	Steffen et al. (2002b)
4.	*Armillaria mellea* (honey fungus, bootlace fungus)	Marasmiaceae	MnP/Lac	Robene-Soustrade et al. (1992)
5.	*Armillaria ostoyae*	Marasmiaceae	MnP/Lac	Robene-Soustrade et al. (1992)
6.	*Auricularia* sp. (wood ear, Jew's-ear)	Auriculariaceae	MnP/Lac	Hofrichter and Fritsche (1997) and Saparrat et al. (2002),
7.	*Bjerkandera adusta*	Hapalopilaceae	LiP/MnP	Pickard et al. (1999), Moreira et al. (2000a, b), and Romero et al. (2007)
8.	*Ceriporiopsis subvermispora*	Hapalopilaceae	MnP/Lac	Lobos et al. (1994), Okano et al. (2005), Mendonça et al. (2008) and Arora et al. (2011)
9.	*Cerrena unicolor*	Polyporaceae	Lac	Gianfreda et al. (1998)
10.	*Cerrena maxima*	Polyporaceae	Lac	Koroleva et al. (2002a) and Elisashvili et al. (2008)
11.	*Chrysonilia sitophila*	–	LiP/MnP	Ferrer et al. (1992)
12.	*Clitocybula dusenii*	Tricholomataceae	MnP/Lac	Nuske et al. (2002)
13.	*Collybia dryophila*	Tricholomataceae	MnP/Lac	Steffen et al. (2002a)
14.	*Coriolopsis gallica*	Polyporaceae	Lac	Pickard et al. (1999)
15.	*Coriolopsis occidentalis*	Polyporaceae	LiP	Nerud and Misurcova (1989)
16.	*Coriolopsis polyzona*	Polyporaceae	MnP/Lac	Nerud et al. (1991) and Elisashvili et al. (2008)
17.	*Coriolopsis rigida*	Polyporaceae	MnP/Lac	Capeleri and Zadrazil (1997) and Saparrat et al. (2002)
18.	*Cyathus bulleri*	Nidulariaceae	LiP/MnP/Lac	Dhawan and Kuhad (2002)
19.	*Cyathus stercoreus*	Nidulariaceae	MnP/Lac	Orth et al. (1993) and Dhawan and Kuhad (2002)
20.	*Daedalea flavida*	–	Lac	Arora and Gill (2001) and, Arora et al. (2002)
21.	*Daedaleopsis confragosa*	Polyporaceae	LiP/MnP	De Jong et al. (1992)
22.	*Dichomitus squalens* (red rot)	Polyporaceae	MnP/Lac	Nerud et al. (1991), Orth et al. (1993) and Arora et al. (2002)
23.	*Fomes fomentarius*	Polyporaceae	LiP	Varela et al. (2000)
24.	*Fomes lignosus*	Polyporaceae	LiP	Huoponen et al. (1990) and Waldner et al. (1988)
25.	*Fomes sclerodermeus*	Polyporaceae	MnP/Lac	Papinutti et al. (2003)
26.	*Ganoderma applanatum*	Ganodermataceae	LiP	Varela et al. (2000)
27.	*Ganoderma australe*	Ganodermataceae	LiP	Varela et al. (2000) and Mendonça et al. (2008)
28.	*Ganoderma lucidum*	Ganodermataceae	LiP/MnP/Lac	Orth et al. (1993), D'Souza et al. (1999), and Han et al. (2005)
29.	*Ganoderma valesiacum*	Ganodermataceae	MnP/Lac	Nerud et al. (1991)
30.	*Grifola frondosa*	Meripilaceae	MnP/Lac	Orth et al. (1993)
31.	*Inonotus hispidus*	Hymenochaetaceae	MnP/Lac	Aggelis et al. (2002)
32.	*Irpex lacteus*	Steccherinaceae	LiP/MnP/Lac	Novotny et al. (2000)

(continued)

Table 1.3 (continued)

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
33.	*Junghuhnia separabilima*	–	LiP/Lac	Vares et al. (1992)
34.	*Lentinula edodes* (shiitake)	Marasmiaceae	MnP/Lac	Orth et al. (1993), Hatvani and Mecs (2001), Nagai et al. (2003) and Okano et al. (2005)
35.	*Lentinus degener*	Polyporaceae	LiP	Varela et al. (2000)
36.	*Lentinus tigrinus*	Polyporaceae	MnP/Lac	Moreira et al. (2000c) and Aggelis et al. (2002)
37.	*Marasmius quercophilus*	Tricholomataceae	MnP/Lac	Tagger et al. (1998) and Klonowska et al. (2001)
38.	*Mycena galopus*	Tricholomataceae	MnP/Lac	Ghosh et al. (2003)
39.	*Nematoloma frowardii*	Strophariaceae	LiP/MnP/Lac	Hofrichter et al. (1999b) and Nuske et al. (2002)
40.	*Oudemansiella radicata*	Marasmiaceae	LiP/Lac	Hüttermann et al. (1989)
41.	*Panaeolus sphinctrinus*	Bolbitiaceae	MnP/Lac	Heinzkill et al. (1998)
42.	*Panellus stipticus*	Tricholomataceae	MnP/Lac	Aggelis et al. (2002)
43.	*Panus tigrinus*	Tricholomataceae	MnP/Lac	Maltseva et al. (1991) and Fenice et al. (2003)
44.	*Peniophora gigantea*	Peniophoraceae	LiP	Varela et al. (2000)
45.	*Perenniporia medulla-panis*	Polyporaceae	MnP	Orth et al. (1993)
46.	*Phanerochaete sordida*	Phanerochaetaceae	MnP	Moreira et al. (2000c)
47.	*Phanerochaete chrysosporium*	Phanerochaetaceae	LiP/MnP	Glenn et al. (1983), Tien and Kirk (1983), Glenn and Gold (1985), Schoemaker and Leisola (1990), Kaal et al. (1995), Gill and Arora (2003), Kuhar et al. (2008) and Zeng et al. (2010)
48.	*Phanerochaete flavido-alba*	Phanerochaetaceae	LiP/MnP	Hamman et al. (1999) and Varela et al. (2000)
49.	*Phellinus gilvus*	Hymenochaetaceae	MnP/Lac	Capeleri and Zadrazil (1997) and Saparrat et al. (2002)
50.	*Phellinus igniarius*	Hymenochaetaceae	Lac	Szklarz et al. (1989)
51.	*Phellinus pini*	Hymenochaetaceae	LiP/MnP	Blanchette et al. (1989) and Bonnarme and Jeffries (1990)
52.	*Phlebia brevispora*	Meruliaceae	LiP/MnP/Lac	Ruttimann et al. (1992), Arora and Rampal (2002) and Arora et al. (2011)
53.	*Phlebia fascicularia*	Meruliaceae	Lac	Arora and Gill (2001), Arora and Rampal (2002), Arora et al. (2002) and Arora et al. (2011)
54.	*Phlebia floridensis*	Meruliaceae	Lac	Arora and Gill (2001), Arora and Rampal (2002), Arora et al. (2002) and Arora et al. (2011)
55.	*Phlebia ochraceofulva*	Meruliaceae	LiP/Lac	Vares et al. (1993)
56.	*Phlebia radiata*	Meruliaceae	LiP/MnP/Lac	Hatakka et al. (1987), Saloheimo et al. (1989), Niku-Paavola et al. (1990), Moreira et al. (2000a, c), Arora and Gill (2001), Arora and Rampal (2002) and Arora et al. (2011)
57.	*Phlebia subserialis*	Meruliaceae	MnP	Bonnarme and Jeffries (1990)
58.	*Phlebia tremellosa*	Meruliaceae	LiP/MnP/Lac	Ralph et al. (1996), Vares et al. (1994) and Robinson et al. (2001)

(continued)

Table 1.3 (continued)

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
59.	*Pholiota aegerita*	Bolbitiaceae	Lac	Von Hunolstein et al. (1986)
60.	*Pleurotus eryngii*	Pleurotaceae	LiP/MnP	Orth et al. (1993), Munoz et al. (1997a, b), Heinfling et al. (1998) and Okano et al. (2005)
61.	*Pleurotus florida*	Pleurotaceae	LiP/Lac	Hüttermann et al. (1989) and Das et al. (1999)
62.	*Pleurotus ostreatus* (oyster mushroom)	Pleurotaceae	LiP/MnP/Lac	Waldner et al. (1988), Palmieri et al. (1997), Reddy et al. (2003), Kurt and Buyukalaca (2010) and Shrivastava et al. (2011)
63.	*Pleurotus pulmonarius*	Pleurotaceae	MnP/Lac	Orth et al. (1993), D'Souza et al. (1996) and Valdez et al. (2008)
64.	*Pleurotus sajor-caju*	Pleurotaceae	LiP/MnP/Lac	Hatakka (1994), Chagas and Durrant (2001), Reddy et al. (2003) and Kurt and Buyukalaca (2010)
65.	*Pleurotus sapidus*	Pleurotaceae	MnP/Lac	Orth et al. (1993)
66.	*Polyporus brumalis*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
67.	*Polyporus ciliatus*	Polyporaceae	MnP/Lac	Moreira et al. (2000c)
68.	*Polyporus osteiformis* (brown-rot fungus)	Polyporaceae	LiP	Dey et al. (1994)
69.	*Polyporus pinsitus*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
70.	*Polyporus platensis*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
71.	*Polyporus varius*	Polyporaceae	LiP	Hüttermann et al. (1989)
72.	*Polyporus versicolor*	Polyporaceae	MnP	Orth et al. (1993)
73.	*Pycnoporus cinnabarinus*	Polyporaceae	Lac	Eggert et al. (1996), Lomascolo et al. (2002) and Kuhar et al. (2008)
74.	*Pycnoporus sanguineus*	Polyporaceae	LiP/MnP/Lac	Pointing et al. (2000), Lomascolo et al. (2002) and Capeleri and Zadrazil (1997)
75.	*Rigidoporus lignosus*	Meripilaceae	MnP/Lac	Galliano et al. (1991) and Cambria et al. (2000)
76.	*Stereum hirsutum*	Stereaceae	MnP/Lac	Nerud et al. (1991)
77.	*Stropharia coronilla*	Strophariaceae	MnP/Lac	Steffen et al. (2002b)
78.	*Stropharia rugosoannulata*	Strophariaceae	MnP/Lac	Steffen (2003)
79.	*Trametes cingulata*	Polyporaceae	MnP/Lac	Orth et al. (1993) and Tekere et al. (2001)
80.	*Trametes elegans*	Polyporaceae	MnP/Lac	Tekere et al. (2001)
81.	*Trametes gibbosa*	Polyporaceae	LiP/MnP/Lac	Nerud et al. (1991)
82.	*Trametes hirsuta* (hairy stereum)	Polyporaceae	LiP/MnP/Lac	Nerud et al. (1991) and Koroleva et al. (2002)
83.	*Trametes pocas*	Polyporaceae	MnP/Lac	Tekere et al. (2001)
84.	*Trametes trogii*	Polyporaceae	LiP/MnP/Lac	Levin et al. (2001, 2002)
85.	*Trametes versicolor* (turkey tail)	Polyporaceae	LiP/MnP/Lac	Blanchette et al. (1989), Huoponen et al. (1990), Black and Reddy (1991), Hatakka (1994), Couto et al. (2002), Gill and Arora (2003) and Shrivastava et al. (2011)
86.	*Trametes villosa*	Polyporaceae	MnP	De Jong et al. (1992) and Capeleri and Zadrazil (1997)
87.	*Volvariella volvacea*	Pluteaceae	Lac	Chen et al. (2004) and Akinyele et al. (2011)

nonselective or simultaneous rot (Blanchette 1995). Some white-rot fungi preferentially remove lignin without a substantial loss of cellulose and cause white-pocket or white-mottled type of rot, e.g., *Phellinus nigrolimitatus* (Blanchette 1995). There are fungi that are also able to produce both types of attack in the same wood (Eriksson et al. 1990). Typical examples of such fungi are *Ganoderma applanatum* and *Heterobasidion annosum*. Because selectively lignin degrading fungi are considered the most promising ones for applications in pulp and paper industry and animal feed development, the search for potent selective lignin degraders has attained a considerable interest. However, the ratio of lignin-hemicellulose-cellulose decayed by a selected fungus can differ enormously, and even different strains of the same species, e.g., of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, may behave differently on the same kind of wood. The lignin-degrading systems of these fungi are important to study since they are very efficient. *C. subvermispora* may be considered as a model fungus for selective lignin degradation.

Other Ascomycetes have also been described to produce laccase and other lignocellulolytic enzymes, which include *Rhizoctonia solani* (Wahleithner et al. 1996), *Aspergillus nidulans* (Clutterbuck 1972), *Podospora anserina, Neurospora crassa* (Tamaru and Inoue 1989), *Gaeumannomyces graminis* var. *tritici* (Edens et al. 1999), and *Trichoderma reesei* (Nieves et al. 1998). The role of Ascomycetous fungi in wood degradation has to be explored. Basidiomycotina attack either hardwood or softwood, while Ascomycotina probably degrade only hardwood (Kirk and Farrell 1987).

Overlapping habits have been found in the three main ecophysiological groups of fungi such as wood-decaying, mycorrhiza-forming, and litter-decomposing fungi (LDF). Wood-decay fungi such as *Hypholoma* sp. (*Nematoloma* sp.), *Pleurotus* sp., and *Armillaria* sp. are also capable of colonizing soil in contact with wood debris (Dix and Webster 1995). Other litter-decomposing fungi can also grow on straw (e.g., *Stropharia rugosoannulata*) that is favored only by wood-decay fungi (Haselwandter et al. 1990). *Volvariella volvacea* is a litter-decomposing fungus belonging to Pluteaceae family but secretes ligninolytic enzymes (laccase) (Chen et al. 2004). *Agaricus bisporus* is also LDF which secretes laccases and manganese peroxidases (Bonnen et al. 1994). The ability to break down lignin and cellulose enables some of the LDF to function as typical white-rot fungi in soil (Hofrichter 2002). Diversity of all these groups of fungi is broad and hence explains their importance in the carbon cycle (Dix and Webster 1995).

Lignin-Degrading Enzymes

Lignin does not contain hydrolysable linkages, which means that the enzymes must be oxidative in nature to degrade it. Lignin is stereoirregular, which also points to more nonspecific attack compared to many other natural polymers. Lignin degradation by white-rot fungi is an oxidative process, and primarily three enzymes, i.e., manganese peroxidase (MnP), E.C. 1.11.1.13; lignin peroxidase (LiP), E.C. 1.11.1.14; and laccases, E.C. 1.10.3.2, are majorly responsible for lignin degradation (Kuhad et al. 1997). However, there are many other enzymes participating in lignin degradation (Sánchez 2009) (Table 1.4). When wood-degrading fungi colonize wood, their extracellular enzymes are not able to diffuse into intact wood cell walls because the enzymes are too large to penetrate the pores of the wood cell walls. Hydroxyl radicals (•OH), a radical species highly destructive for cellulose and lignin, are proposed as a principal low molecular mass oxidant that erodes wood cell walls to enhance the accessibility of the extracellular enzymes of wood-rot fungi to wood cell wall components (Wood 1994).

LiP and MnP oxidize the substrate by two consecutive one-electron oxidation steps with intermediate cation radical formation. Laccase has broad substrate specificity and oxidizes phenols and lignin substructures with the formation of oxygen radicals. Other enzymes that participate in the lignin degradation processes are H_2O_2-producing enzymes and oxidoreductases, which can be located either intracellularly or produced extracellularly.

Table 1.4 Extracellular ligninolytic enzymes involved in lignin degradation

S. No.	Enzyme	Cofactor	Substrate/mediator	Main effect or reaction
1.	Lignin peroxidase (LiP)	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to cation radical
2.	Manganese peroxidase (MnP)	H_2O_2 Mn^{2+}	Organic acids	Chelators, thiols and unsaturated lipids Mn^{2+} oxidized to Mn^{3+}; further oxidation of phenolic compounds to phenoxy radicals
3.	Laccase (Lac)	O_2	Hydroxybenzotriazole, ABTS	Phenols are oxidized to phenoxyl radicals; mediator radicals
4.	Versatile peroxidases (VP)		H_2O_2	Same or similar compounds as LiP and MnP. Same effect on aromatic and phenolic compounds as LiP and MnP
5.	Aryl alcohol oxidase (AAO)	–	Aromatic alcohols (anisyl, veratryl alcohol)	O_2 reduced to H_2O_2
6.	Cellobiose quinone 1-oxidoreductase (CBQ)	–	–	Cellobiose reduced to o- and p-quinones

The accessibility of cell wall polysaccharides from the plant to microbial enzymes is dictated by the degree to which they are associated with phenolic polymers (Chesson 1981; Kuhad et al. 1997). Several white-rot fungi such as *P. chrysosporium, P. ostreatus, T. versicolor, C. subvermispora,* and *P. cinnabarinus* have been studied in more detail for their ligninolytic system (Table 1.3). Some strains of white-rot fungi produce all the three well-characterized enzymes, while others produce either two or one of them. For details, please see Table 1.3. The model fungus for lignin degradation is *P. chrysosporium;* however, recently certain other lignin-degrading fungi have also been studied for lignin degradation, viz., C. *subvermispora*, *P. radiata*, *P. eryngii, C. bulleri, P. ostreatus, and T. versicolor* (Kirk 1990; Lundell 1993; Martinez et al. 1994; Hatakka 2001; Vasdev et al. 2005; Okano et al. 2009; Shrivastava et al. 2011).

Lignin-degrading enzymes are produced during the secondary metabolism of white rots. Since lignin oxidation does not provide net energy to the fungus, synthesis and secretion of these enzymes is often induced by limited nutrient levels (mostly C or N). Production of LiP and MnP is generally optimal at high oxygen tension but is repressed by agitation in submerged liquid culture, while laccase production is often repressed by agitation; however, it varies from fungus to fungus (Vasdev and Kuhad 1994). Production of laccase by solid-state fermentation has been reported for *P. cinnabarinus*, which was grown on sugarcane bagasse packed in 250-mL columns (Lomascola et al. 2003). Banana waste can also be used as a substrate for laccase production by *Aspergillus* sp. (Shah et al. 2005). *P. sanguineus* produced laccase on three more substrates under SSF conditions which are sago "hampas," rubberwood sawdust, and oil palm from parenchyma tissue (OPFPt) (Vikineswary et al. 2006). More recently production of LiP, MnP, and laccase has been reported using various substrates and organisms, i.e., *P. ostreatus* on wheat straw; *Aspergillus sclerotiorum*, *Cladosporium cladosporioides,* and *Mucor racemosus* in salinity conditions with wheat bran; and *P. chrysosporium* on compost (Robertson et al. 2008; Bonugli-Santos et al. 2010; Zeng et al. 2010). The ligninolytic enzymes have varied properties, and their discussion will be beyond the scope of this chapter; however, some properties are given in Table 1.5.

Lignin-degrading enzymes are essential for lignin degradation; however, for lignin mineralization, the role of some additional enzymes is equally significant. Such auxiliary enzymes (by themselves unable to degrade lignin) are glyoxal oxidase and superoxide dismutase for

Table 1.5 Some properties of MnP, LiP, and Laccase

E.C. No:	MnP 1.11.1.13	LiP 1.11.1.14	Lac 1.10.3.2
Cofactor	Mn(II): H_2O_2	Diarylpropan O_2, H_2O_2	p-Benzendiol: O_2-
Class	Oxidoreductases	Oxidoreductases	Oxidoreductases
Prosthetic group	Heme	Heme	type 1 Cu, type 2 Cu, type 3 Cu
MW (kDa)	32–62.5 (122)	38–47	59–110 (tetramers V 390c)
Glycosylation	N-type	N-type	N-type
Isoforms	Monomers; up to 11	Monomers; up to 15	Mono-, di-, tetramers; several
p*I*	2.8–7.2	3.2–4.7	2.6–4.5
pH range	2.6–4.5	2.0–5.0	2.0–8.5
E° (mV)	1,510	1,450	500–800
C–C cleavage	Yes	Yes	No
H_2O_2 regulated	Yes	Yes	No
Stability	+ + +	+	+ + +
Native mediators	Mn^{2+}; Mn^{3+} Mn^{2+}	VA, 2Cl-14DMB	3-HAA
Specificity	Mn^{2+}	Broad, aromatics, including nonphenolics	Broad, phenolics
Secondary and synthetic mediators	Thiols, unsaturated fatty acids	No	ABTS, HBT, syringaldazine

Modified from Fakoussa and Hofrichter (1999)
ABTS 2′,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), *VA* veratryl alcohol, *HAA* 3-hydroxy-antranilic acid, *DMB* 2,6-dimethyl-1,4-benzoquinone, *HBT* 1-hydroxybenzotriazole

intracellular production of H_2O_2, a cosubstrate of LiP and MnP, as well as glucose oxidase, aryl alcohol oxidase, and cellobiose dehydrogenase (CDH) involved in feedback circuits and linking ligninolysis with cellulose and hemicellulose degradation in nature (Leonowicz et al. 1999). Lignin has been found to be partly mineralized in cell-free system of lignin-degrading enzyme, with considerably enhanced rates in the presence of co-oxidants such as fatty acids or thiols (Hofrichter et al. 1998).

Lignin Peroxidase (LiP)

LiP (EC 1.11.1.14) is an extracellular heme-containing peroxidase which is dependent on H_2O_2 and has an unusually high redox potential and low optimum pH (Bonugli-Santos et al. 2010), typically showing little specificity toward substrates and degrades a variety of lignin-related compounds (Barr and Aust 1994). It has molecular masses between 38 and 47 kDa (Table 1.5) (Tien et al. 1986). LiP is well known as part of the ligninolytic system both of aphyllophoralic and agaricalic fungi (Glenn et al. 1983; Hatakka et al. 1987; Hofrichter and Fritsche 1997). Lignin peroxidases (LiP) of *P. chrysosporium* are encoded by a family of six closely related genes (Stewart et al. 1992). More than ten heme proteins displaying ligninolytic activity have been detected in the extracellular fluid of cultures of *P. chrysosporium* BKM-F-1767, and they are designated H1–H10 (Rothschild et al. 1997)

LiP preferably oxidizes methoxylated aromatic ring without a free phenolic group, such as the model compound dimethoxybenzene (Kersten et al. 1990). Thus, the cleavage of Cα–Cα bonds is catalyzed preferentially in dimeric nonphenolic lignin model compounds (Kuhad et al. 1997). LiP oxidizes target substrates by two one-electron oxidation steps with intermediate cation radical formation (Sánchez 2009; Dashtban et al. 2010) (Fig. 1.1). The simplest aromatic substrates for LiP are methoxylated benzenes and benzyl alcohols, which have been used extensively by enzymologists to study LiP reaction mechanisms. The H_2O_2-dependent oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) to veratraldehyde is the basis for the standard assay used to detect LiP

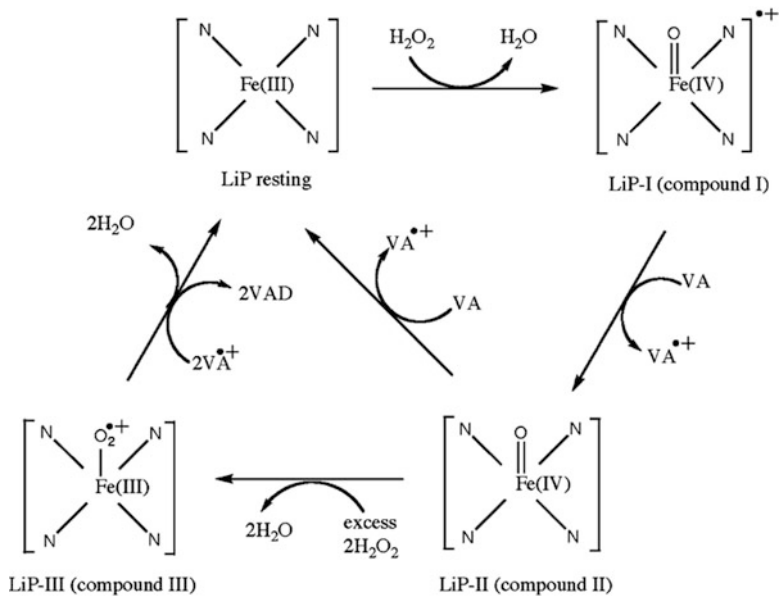

Fig. 1.1 Catalytic cycle of lignin peroxidase (Source: Wong 2009)

in fungal cultures (Kirk 1990). The role of LiP in ligninolysis could be further transformation of lignin fragments which are initially released by MnP. Studies have shown that LiP may not be essential for the attack on lignin, as several highly active WRF and litter-decaying fungi (e.g., *C. subvermispora, Dichomitus squalens, P. tigrinus, R. lignosus*) do not excrete this enzyme (Galliano et al. 1991; Maltseva et al. 1991; Périé and Gold 1991; Hatakka 1994). LiP has been used to mineralize a variety of recalcitrant aromatic compounds, such as three- and four-ring polyaromatic hydrocarbons, polychlorinated biphenyls, and dyes (Chivukula et al. 1995; Gunther et al. 1998; Krcmar and Ulrich 1998). 2-Chloro-1, 4-dimethoxybenzene, a natural metabolite of white-rot fungi, is reported to act as a redox mediator in the LiP-catalyzed oxidations (Teunissen and Field 1998).

Manganese Peroxidase (MnP)

MnP (EC 1.11.1.13), an extracellular heme-containing peroxidase with a requirement for Mn^{2+} as its reducing substrate, was first discovered shortly after LiP from *P. chrysosporium* by Kuwahara et al. (1984) and simultaneously described by Glenn and Gold (1985). MnP is one of the most common lignin-degrading peroxidases produced by the majority of wood-decaying fungi and by many litter-decomposing fungi (Hofrichter 2002). These are glycosylated proteins with an iron protoporphyrin IX (heme) prosthetic group (Glenn and Gold 1985). The molecular weights range between 32 and 62.5 kDa, and these are secreted in multiple isoforms (Table 1.5) (Urzúa et al. 1995; Hofrichter 2002; Boer et al. 2006). MnP oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes phenolic structures to phenoxyl radicals (Gold et al. 1989). Mn^{3+} formed is highly reactive and complexes with chelating organic acids such as oxalate or malate (Cui and Dolphin 1990; Kishi et al. 1994), which are produced by *P. radiata* (Galkin et al. 1998; Hofrichter et al. 1999b). With the help of these chelators, Mn^{3+} ions are stabilized and can diffuse into materials such as wood. The redox potential of the MnP-Mn system is lower than that of LiP, and these preferably oxidizes phenolic substrates (Vares 1996). The phenoxyl radicals produced can further react with the eventual release of CO_2.

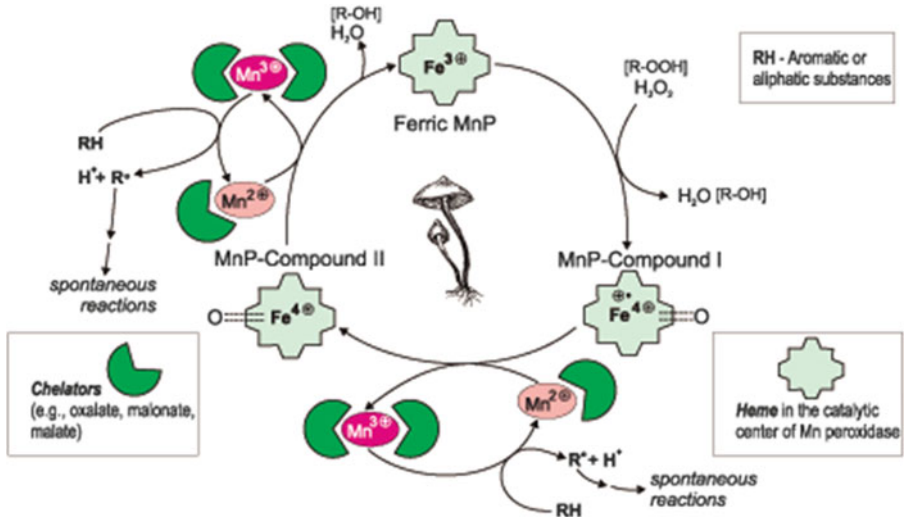

Fig. 1.2 The catalytic cycle of MnP (Redrawn from Wariishi et al. 1989; Hofrichter 2002)

The catalytic cycle of MnP is reviewed in detail by Kuhad et al. (1997), and is very similar to that of LiP, differing only in that compound II is readily reduced by Mn^{2+} to its native form (Fig. 1.2) (Wariishi et al. 1989; Sánchez 2009). The phenoxyl radicals formed subsequently cleave Cα-Cα or alkyl-phenyl bonds causing depolymerization to smaller intermediates including quinones and hydroxyl quinones (Kuhad et al. 1997). Purified or crude MnP has been used in cell-free systems (in vitro) and shown to oxidize not only lignin (Hofrichter et al. 1999a, 2001) but also chlorolignins (Lackner et al. 1991) and synthetic lignin compounds (Hofrichter et al. 1999b, c; Cullen and Kersten 2004).

Laccase

Laccases (EC 1.10.3.2) are widespread in nature, and in fungi they are implicated in pigmentation (Clutterbuck 1972; O'Hara and Timberlake 1989), fruit body formation (Leatham and Stahman 1981), and pathogenicity (Choi et al. 1992), as well as in lignin degradation (Sigoillot et al. 2005; Dashtban et al. 2010). Fungal laccases as part of the ligninolytic enzyme system are produced by almost all wood and litter-transforming Basidiomycetes and some Ascomycetes; however, their levels differ (Claus 2003). This group of N-glycosylated extracellular blue oxidases with molecular mass of 40–90 kDa contains four copper atoms in the active site that are distributed among different binding sites and are classified into three types with differential specific characteristic properties one type 1, one type 2, and two type 3 (Table 1.5) (Reinhammar 1984; Call and Mucke 1997; McGuirl and Dooley 1999; Claus 2003; Claus and Decker 2006). All these copper ions are apparently involved in the catalytic mechanism. Fungal laccases of different origin have shown some divergence in copper atom number and their spectrum character. Type 3 binuclear copper is absent in both L2 from *P. florida* and the laccase from *P. eryngii* (Munoz et al. 1997a, b), while type 1 copper is absent in laccase from *Phellinus ribis* and *P. ostreatus* (Palmieri et al. 1997).

Laccases catalyze the oxidation of a variety of aromatic hydrogen donors with concomitant reduction of oxygen to water (Fig. 1.3). Moreover, laccases do not only oxidize phenolic and methoxyphenolic acids but also decarboxylate them and attack their methoxy groups (demethylation). Until 1990, laccase had been considered to be able to degrade only phenolic compounds (Higuchi 1989). However, Bourbonnais and Paice (1990) reported first time that laccase can

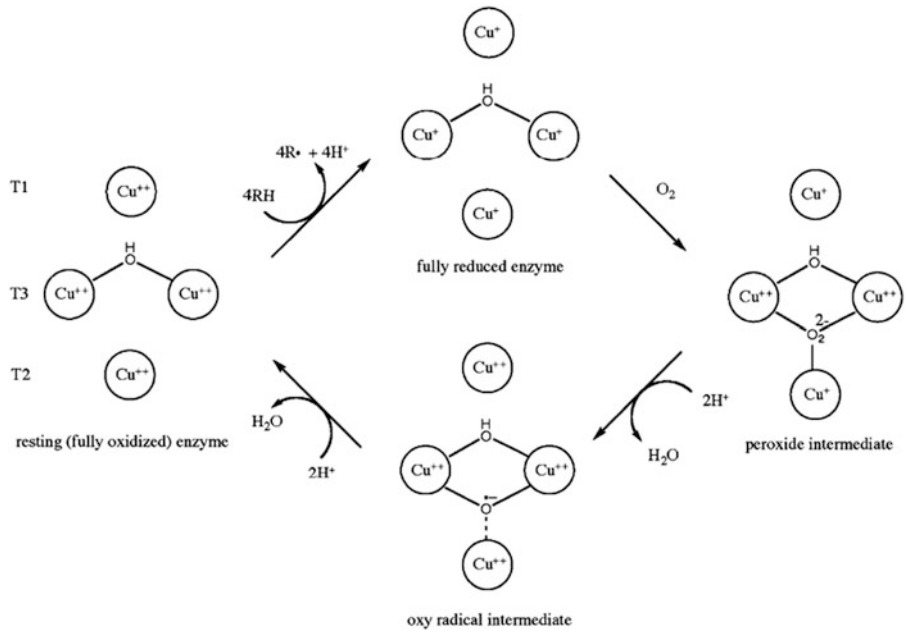

Fig. 1.3 Typical reaction of laccase (Source: Wong 2009)

also oxidize nonphenolic compounds in the presence of a suitable redox mediator such as ABTS 2′,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate). Recently, Riva (2006) has shown the laccase-catalyzed redox cycles for substrate oxidation in the presence and absence of mediators (Fig. 1.4).

Several fungal laccases have been compared for the oxidation of a nonphenolic lignin dimer, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy) propan-1,3-diol (I), and a phenolic lignin model compound, phenol red, in the presence of the redox mediators 1-hydroxybenzotriazole (1-HBT) and violuric acid (Li et al. 1998). Laccases have been reported to oxidize many recalcitrant substances, such as chlorophenols (Ricotta et al. 1996; Grey et al. 1998; Fahr et al. 1999), polycyclic aromatic hydrocarbons (PAHs) (Majcherczyk et al. 1998; Duran and Esposito 2000; Pointing 2001), lignin-related structures (Bourbonnais et al. 1996), and organophosphorous compounds (Amitai et al. 1998; Couto and Herrera 2006), probably following one of the reactions shown in Fig. 1.4. Recently, laccase production has been successfully reported using distillery spent wash and lignocellulosic biomass by *Aspergillus heteromorphus* (Singh et al. 2010).

However, commercial application of laccase faces major obstacles such as the lack of sufficient enzyme stocks and the cost of redox mediators. Heterologous expression of the enzymes with protein engineering may allow for the cost-effective creation of more robust and active enzyme (Dashtban et al. 2010). The development of molecular techniques applied in the fungus has now allowed the identification of regulators of laccase by methods such as insertional mutagenesis and complementation of mutants (Sharma and Kuhad 2010).

Other Lignin-Degrading Enzymes

For complete decomposition and partial mineralization of plant lignocellulose, additional fungal enzymes are required. On the contrary to cellulose, lignin does not supply a carbon or energy source for the wood-decaying fungi (Kirk and Farrell 1987). Another important enzyme called versatile peroxidase (VP) has been recently recognized that can be regarded as hybrid between MnP and LiP, since it can oxidize not only Mn^{2+}

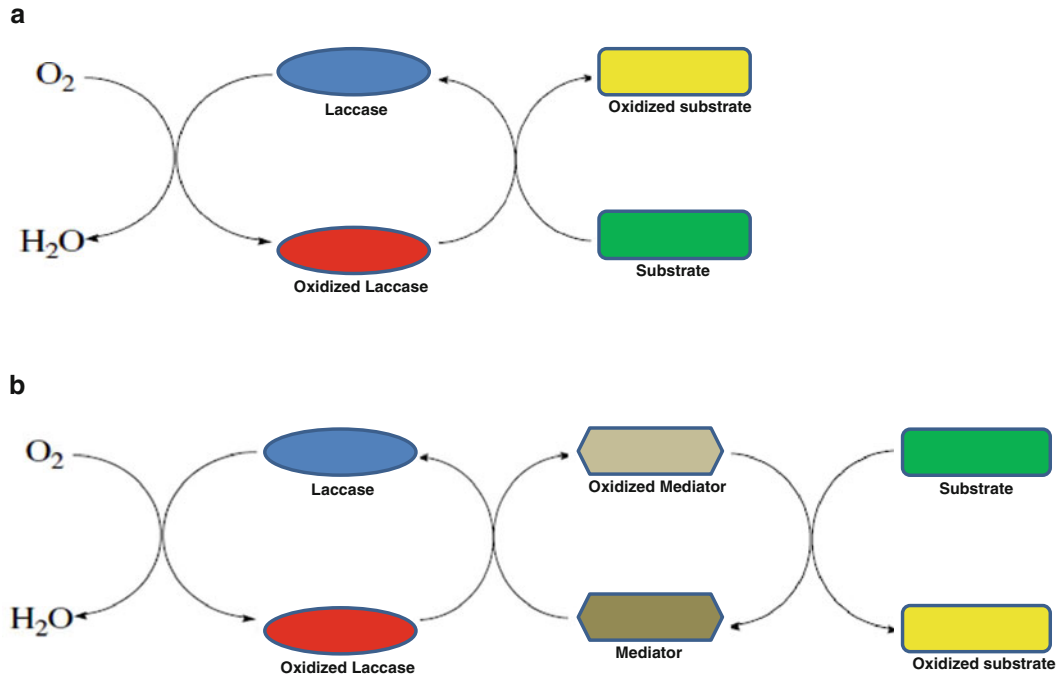

Fig. 1.4 Laccase-catalyzed redox cycles for substrates oxidation in the absence (**a**) or in the presence (**b**) of mediator (Modified from Kunamneni et al. 2008)

but also phenolic and nonphenolic aromatic compounds including dyes. It has been suggested that VPs can oxidize substrate spanning a wide range of potentials, including low- and high-range potentials (Dashtban et al. 2010). This is a result of their hybrid molecular structures which provide multiple binding sites for the substrates (Camarero et al. 1999). VP has been reported in species belonging to *Pleurotus* and *Bjerkandera* (Heinfling et al. 1998; Mester and Field 1998). White-rot fungi possess a variety of oxidative enzymes (Table 1.4), which are capable of generating H_2O_2 (required by peroxidases), through the oxidation of different substrates. Glyoxal oxidase (GLOX; EC 1.2.3.5) and aryl alcohol oxidase (AAO; EC 1.1.3.7) are both extracellular enzymes which were first described by Kersten and Kirk (1987) and later by others (Waldner et al. 1988; Muheim et al. 1990). They use either glyoxal or aromatic alcohols as substrate (Hatakka 2001). In particular AAO is involved in the selective degradation of lignin by *Pleurotus* species (Martinez et al. 1994).

Moreover, intracellular enzymes such as glucose oxidase and pyranose oxidase are also produced by various basidiomycetous fungi, which in turn make H_2O_2 available to the fungus for lignin degradation (Kuhad et al. 1997; Volc et al. 2001). For control of the organic acid metabolism, the mainly intracellular, Mn-containing enzymes oxalate decarboxylase (ODC, EC 4.1.1.2) and oxalate oxidase (EC 1.2.3.4), and the NAD^+-dependent enzyme formate dehydrogenase (EC 1.2.1.2) play important roles in the wood-decaying fungi (Micales 1997; Mäkelä et al. 2002, 2009). One fungal enzyme connecting cellulose decomposition to lignin decay is the extracellular cellobiose dehydrogenase (CDH, EC 1.1.99.18), of which the quinone-reducing cellobiose oxidase is a proteolytical cleavage product (Henriksson et al. 2000). This enzyme was early on described in, e.g., *P. chrysosporium,* and is able to use cellobiose as reducing substrate while donating electrons to quinones and oxidized phenolic intermediates, which may be formed during lignin decomposition (Bao et al. 1993).

Delignification of Lignocellulosic Materials and Their Bioconversion in Digestible and Nutritive Animal Feed

Lignocellulosic materials are the most abundant renewable organic compounds. Since they are major products from agriculture, forestry, urban refuge, and food wastes, tremendous quantities of inexpensive materials are potentially available for use as substrates in fermentation media (Akinyele et al. 2011). Crop residues constitute about 123×10^6 t year^{-1} of this renewable resource, containing approximately 60% cellulose and hemicellulose and 30% lignin on dry mass basis (Bhatnagar et al. 2008). The close association of hemicellulose and cellulose with lignin makes the carbohydrates of plant materials less accessible to microorganisms and enzymes.

Methods to remove lignin partially or completely would offer a means of increasing palatability, digestibility, and nutritional value of plant residues. Several physical treatments, such as pelleting, grinding, steaming, and irradiation as well as physical and chemical treatments, particularly use of ammonia fiber expansion and alkali, have been tested to improve the dry matter digestibility and intake of straw (Walker 1984; Sundstol and Owen 1984; Yadav and Tripathi 1991; Sirohi and Rai 1999; Abdullah et al. 2004; Kristensen et al. 2008; Bals et al. 2010). The practical use of the treatments is limited by safety concern, costs, and serious environmental concerns (Kuhar et al. 2008). The biological treatment may offer a practical and environment friendly alternative to nutritionally upgrade the low-quality roughage. Lignin is degraded to different extents by various microorganisms, of which wood-rot fungi are the most effective, white-rot fungi in particular (Kirk and Farrell 1987; Eriksson et al. 1990; Kuhad et al. 1997; Villas-Boas et al. 2002; Okano et al. 2005, 2009; Shrivastava et al. 2011). The common pattern of attack on plant material by white-rot fungi is simultaneous decay of polysaccharides (cellulose and hemicellulose) and lignin, but preferential degradation of lignin may also occur (Blanchette et al. 1992; Akin et al. 1993b; Kuhad et al. 1997; Eggert et al. 1998; Dhawan and Kuhad 2002; Okano et al. 2005; Kuhar et al. 2008; Shrivastava et al. 2011). Pattern and extent of lignin removal vary, however, for different fungal species and even for strains of a species. Some white-rot fungi selectively degrade lignin without affecting much of the carbohydrates, thus exposing protected and available carbohydrates, which is a prerequisite for animal feed development (Basu et al. 2002).

Considering the availability of the crop residues and grasses, considerable efforts have been made to upgrade their nutritive value (Flachowsky et al. 1999; Basu et al. 2002; Okano et al. 2005; Shrivastava et al. 2011).

Since more than a century, wood delignified by *Ganoderma australe* and other microorganisms was traditionally used as cattle feed in Southern Chile and is called Palo Podrido (Phillippi 1893). Moreover, most of the reported research has dealt with delignification of wood by white-rot fungi, of which *P. chrysosporium* is most studied (Eriksson et al. 1990; Kirk and Cullen 1998; Basu et al. 2002; Kumar and Gomes 2008; Gassara et al. 2010). However, many investigations have been carried out simultaneously to microbially delignify herbaceous plants biomass to improve the utilization of lignocellulosics by ruminating animals. In the similar context a great amount of work has been done at laboratory scale for the utilization of lignocellulosic residues for microbial protein and feed production using fungi such as *Chaetomium cellulolyticum, Aspergillus terreus, Trichoderma viride, Aspergillus niger, Sporotrichum pulverulentum (Phanerochaete chrysosporium), Fusarium oxysporum, T. koningii, Agrocybe aegerita, Ganoderma lucidum, Fusarium concolor, Volvariella volvacea, and Pleurotus ostreatus* (Moo-Young et al. 1979; Chahal et al. 1979; Ek and Eriksson 1975, 1980; Garg and Neelakantan 1982; Hatakka and Pirhonen 1985; Kahlon 1986; Singh et al. 1991; Zadrazil and Puniya 1995; Adamovic et al. 1998; Han et al. 2005; Li et al. 2008; Akinyele et al. 2011).

Research initiated in 1970 at the Central American Research Institute, Guatemala, on utilization of coffee wastes, was later evaluated

in a pilot plant with two 20,000 L fermenters (Aguirre et al. 1976). By growing two fungal strains, *Trichoderma harzianum* and a *Verticillium* sp. under non-sterile conditions, an average 62% reduction of COD of the waste was achieved. The dry mass yield was about 0.4 g g^{-1} carbohydrate, with a net increase of 3–3.5 g of cell mass L^{-1} attained after 24 h. Amino acid analysis of the product revealed a typical fungal protein profile, with limited methionine and high lysine content.

A process for a direct conversion of solid lignocellulosic wastes to protein by the white-rot fungus *Sporotrichum pulverulentum* (*P. chrysosporium*) was developed at the Swedish Pulp and Paper Research Institute (STFI) (Ek and Eriksson 1980). The fungus chosen for the STFI process is particularly well suited for the purpose of protein production from wood components since it produces enzymes for degradation of all the components in lignocellulosic materials. However, the STFI process was not found to be economically feasible on evaluation, since the protein produced could not compete on a cost basis with soybean protein, unless the substrate used in the fermentation had a negative value.

The STFI approach to protein production based on lignocellulosic material was thus changed to a water purification process, in which dissolved substances in the white water from mechanical pulp production were used as substrates (Eriksson et al. 1990). The water purification and protein production process were operated on a 25-m^3 scale using wastewater from a board factory (Ek and Eriksson 1980). The resulting protein was tested in feeding trials at the Swedish Agricultural University, Uppsala. The feeding trials were essentially positive with ruminants, but monogastric animals could not easily digest the fungal cell walls (Thomke et al. 1980). In spite of the positive feeding trials with ruminants, the production of fodder protein by S. *pulverulentum* (*P. chrysosporium*) was economically feasible only if the cost of alternative water purification is taken into account (Eriksson et al. 1990).

The "Waterloo" process for the production of microbial protein from waste biomass was developed at the University of Waterloo, Ontario, Canada (Moo-Young et al. 1979). The process is based on the cellulolytic fungus *Chaetomium cellulolyticum* and utilizes agricultural residues such as straw, corn stoves, bagasse, and forestry residues such as wood sawdust and pulp mill sludges (Moo-Young et al. 1978). The process uses a three-stage operation involving:

1. Thermal and/*or* Chemical Pretreatment of Lignocellulosic substrate
2. Aerobic Fermentation of the Pretreated Material with Nutrient Supplement
3. Recovery of the microbial protein product

The pretreated substrate is fed (2% w/v) continuously (dilution rate of 0.24 h^{-1}) into a 1,000-L fermenter operating at pH 5.5 and 37 °C. Recovery of fungal mycelium is accomplished by filtration, and the dried product contains about 45% crude protein. Nutrition trials conducted with rats, chicks, and piglets indicated that the product is satisfactory as an animal feed protein in terms of its safety, digestibility, and nutritive value (Touchburn et al. 1986). The process employs low-technology operations and efficient mass and energy exchanges between processing streams. Capital and operational expenditures required for the manufacture of fungal protein product are relatively small compared to those for other microbial protein production processes. This could allow for an economic operation of relatively small-size plants in medium-size villages in developing countries (Moo-Young et al. 1978). Agosin and his coworkers (Agosin et al. 1985) found that nonselective degradation of plants by *P. chrysosporium* resulted in small increase in in vitro digestibility by ruminating microorganisms, while other two fungi tested improved the digestibility by 63 and 94%.

A research group at NDRI, Karnal, India, attempted to study the effect of urea treatment and followed by fermentation of urea-pretreated wheat straw by a white-rot fungus, *Coprinus fimetarius* (now called as *C. cinereus*). This treatment, the so-called Karnal process, was characterized by a 30-day ensiling of rice straw sprayed with urea, inoculated with 30% *C. fimetarius,* and treated for 14 days at 35–45 °C (Gupta 1987). Fungal-treated straw was degraded to a lesser extent than untreated and urea-treated straw.

However, this process could not be improved and commercialized.

Zadrazil (1985) evaluated 235 strains of fungi for their ability to delignify wheat straw and reported extreme variations in activity, with substantial influences by temperature and gases (Zadrazil and Puniya 1995). Jung et al. (1992a) reported both increased and decreased in vitro digestibility of oat straw after pretreatment with different fungi. Further work by these workers with *P. chrysosporium* to delignify grass and legume cell walls resulted in loss of more polysaccharides than lignin and did not result in increase in digestibility by rumen microorganisms under their conditions (Jung et al. 1992b). In the similar context, Moyson and Verachtert (1991) reported *Pleurotus pulmonarius* as the best organism for animal feed production while compared with well-studied organisms, i.e., *Pleurotus sajor-caju* and *Lentinus edodes,* due to its potential for degrading higher lignin in comparison to carbohydrate content.

Similarly, Karunanandaa et al. (1992) have tested various fungi and reported increases and decreases in in vitro ruminal digestibilities of corn and rice straw. They further reported the improvement in the digestibility when the fungi selectively used hemicellulose rather than cellulose. Akin et al. (1993b) have also studied the delignification ability of three wild-type fungi and two cellulase-less mutants of *P. chrysosporium* K-3, and among them *C. subvermispora* caused greatest losses in lignin and improved the biodegradation of Bermuda grass over the control substrates.

"Ideal organisms" for converting agricultural crop residues into animal feed should have a strong lignin metabolism with poor degradation of cellulose and hemicellulose, so that biological treatment could lead to produce cellulose-rich material for the utilization of ruminating microflora as an energy source in ruminants (Moyson and Verachtert 1991; Zadrazil et al. 1996; Basu et al. 2002; Okano et al. 2005; 2009; Shrivastava et al. 2011).

Table 1.6 depicts the ability of various white-rot fungi for upgrading fibrous animal feed. It is apparent from the above-mentioned works that white-rot fungi generally require longer duration to maximize the degradation of lignin. The major problem of biological conversion of lignocellulosics into nutritionally improved animal feed is to select microorganisms capable of degrading the lignin selectively. Suitable microorganism should metabolize lignin efficiently and selectively without much degradation of cellulose (Villas-Boas et al. 2002).

Keeping in view the importance of selective lignin degradation and higher digestibility improvement, SSF process was further attempted to be optimized for obtaining the best possible conditions for animal feed production by various workers. Among them, initially, Tripathi and Yadav (1992) optimized SSF of wheat straw into animal feed by *Pleurotus ostreatus* in terms of its cultural and nutrition conditions, i.e., pH, initial moisture, temperature, incubation period, form of inoculum, substrate turning, urea level, etc.; the fungus behaved optimally under the following set of fermentation conditions: initial pH, 5.5; initial moisture, 55%; temperature, 22 °C; period, 21 days; form of inoculum-grain culture (spawn); substrate-turning frequency, once at mid-incubation; urea (nitrogen source) level, 1% (sterile) or 2% (unsterile); single superphosphate (phosphorus + sulfur source), 0.3%; and no addition of free carbohydrates (as whey or molasses). A maximum (10.4%) increase in IVDMD accompanied by a 2.7% degradation of lignin was attained in the optimized SSF under the above conditions. Furthermore Basu et al. (2002) also studied the effect of seed culture on solid-state bioconversion of wheat straw by *Phanerochaete chrysosporium* for animal feed production under statistical designs based on a central composite experimental design. The conditions of the seed culture most suitable for rapid induction of the ligninolytic activity of the fungus were determined. When the seed culture with an initial pH of 5.8 was grown under agitated conditions at 130 rpm in baffled flasks at 38 °C, it was predicted to give lignin degradation of 19.5% and cellulose degradation of 17.8%. A time profile study of the solid-state bioconversion of wheat straw indicated that the highest lignin and lowest cellulose degradation levels occurred on the sixth

Table 1.6 Modification to composition and rumen digestibility of agricultural waste biomass by white-rot fungi

S. No.	Substrate	Fungus	Period of incubation (days)	Relative change in			References
				Lignin (%)	Cellulose (%)	IVDMD (%)	
1.	Ryegrass	*Candida utilis* (NRRL-Y-1084)	3	+3.8	−11.6	+49.2	Grant et al. (1978)
2.	Wheat straw	*Pleurotus ostreatus*	30	−2.8	−14.9	+8.8	Tripathi and Yadav (1992)
3.	Corn straw	*Lentinus edodes*	49	−43	~0	~67	Sermanni et al. (1994)
4.	Hardwood trunks	*L. edodes*	2 years	1	–	427	Suzuki et al. (1995)
5.	Hardwood sawdust, + rice bran	*Pholiota nameko*	100	−30	–	135	Suzuki et al. (1995)
6.	Maize stover	*Cyathus stercoreus*	28	9	17	28	Chen et al. (1995)
7.	Maize stover	*Phanerochaete chrysosporium*	28	48	−29	−24	Chen et al. (1995)
8.	Rice straw	*C. stercoreus*	30	−53	−3	–	Karunanandaa and Varga (1996)
9.	Wheat straw	*Trametes gibbosa*	30	−27	−7	30	Jalc et al. (1996)
10.	Sugarcane bagasse	*Athelia* sp.	30	−17	9	–	Breccia et al. (1997)
11.	Wheat straw	*Peniophora utriculosa*	30	−55	–	36	Capeleri and Zadrazil (1997)
12.	Wheat straw	*Daedalea quercina*	30	−38	−9	43	Jalc et al. (1997)
13.	Wheat straw	*Inonotus dryophilus*	30	26	1	35	Jalc et al. (1997)
14.	Wheat straw	*Pleurotus* sp.	30	−12.82	−7.82	+6.7	Kakkar and Dhanda (1998)
	Paddy straw	*Pleurotus* sp.	10	−11.96	−7.48	−9.24	Kakkar and Dhanda (1998)
15.	Wheat straw	*Phanerochaete chrysosporium*	8	−19.5	−17.8	–	Basu et al. (2002)
16.	Apple pomace	*Candida utilis*	6	−70	−8.3	+8.2	Villas-Boas et al. (2003)
	Apple pomace	*Pleurotus ostreatus*	30	−40	−11.11	+7.0	Villas-Boas et al. (2003)

17.	Sugarcane bagasse	*Lentinus edodes*	16 weeks	−38.09	–	+51.53	Okano et al. (2006)
	Sugarcane bagasse	*Pleurotus eryngii*	16 weeks	+4.76	–	+5.04	Okano et al. (2006)
	Sugarcane bagasse	*Pleurotus salmoneo stramineus*	16 weeks	+10.47	–	+6.36	Okano et al. (2006)
	Sugarcane bagasse	*Ceriporiopsis subvermispora*	16 weeks	−30.48	–	+34.43	Okano et al. (2006)
18.	Wheat straw	*Phanerochaete chrysosporium*	5	−27.0	−29.0	–	Kumar and Gomes (2008)
19.	Wheat straw	*Phanerochaete chrysosporium*	7	−13 to −37	−25 to 50	–	Bhatnagar et al. (2008)
20.	Paddy straw	*Phanerochaete chrysosporium*	60	−39.4	−52.0	+35.13	Sharma and Arora (2010b)
		Ceriporiopsis subvermispora	60	−18.8	−30.8	+29.73	Sharma and Arora (2010b)
		Phlebia brevispora	60	−20.0	−8.7	+36.22	Sharma and Arora (2010b)
		Pleurotus fascicularia	60	−21.0	−10.3	+28.1	Sharma and Arora (2010b)
		Pleurotus floridensis	60	−21.8	−11.4	+25.4	Sharma and Arora (2010b)
		Pleurotus radiata	60	−222.8	−13.4	+34.05	Sharma and Arora (2010b)
21.	Wheat straw	*Pleurotus ostreatus*	10	−20.52	−15.64	+9.5	Shrivastava et al. (2011)
	Wheat straw	*Trametes versicolor*	10	−7.45	−12.18	+6.12	Shrivastava et al. (2011)
22.	Wheat straw	*Pleurotus brevispora*	30	−30.6	–	+66.80	Arora et al. (2011)
		Pleurotus radiata	30	−27.9	–	~+40	Arora et al. (2011)
		Pleurotus floridensis	30	−27.5	–	~+51.16	Arora et al. (2011)
		Ceriporiopsis subvermispora	30	−25.2	–	~+45.35	Arora et al. (2011)
		Pleurotus fascicularia	30	−23.1	–	~ +56.98	Arora et al. (2011)

+ and – signs indicate % increase and decrease, respectively

day of cultivation. The desirability coefficient for this process also passed through a maximum of 0.705 on the sixth day, which was predicted to be the best time for harvesting the batch.

Several strains of white-rot fungi, i.e., *P. chrysosporium*, *T. versicolor*, *C. stercoreus, C. subvermispora*, and *P. cinnabarinus,* have been largely used as model systems to study lignin degradation. To the best of our knowledge the *C. subvermispora* have been found an efficient lignin degrader when tested under laboratory scale. Kakkar and Dhanda (1998) fed *Pleurotus*-fermented wheat and rice straw to buffalo, and fermentation caused an increase in CP up to 80%, lignin reduction up to 12%, and improvement in dry matter intake and nutrient digestibility in animals. Whereas, Okano et al. (2005) fermented wheat straw, bagasse, and Konark oak with *Pleurotus* and *Lentinus* spp. up to 96 days and 4 years, respectively, and reported both increase and decrease in organic matter digestibility. Recently, in Nigeria, Belewu (2006) has converted Masonia tree sawdust and cotton plant by-product (CBP) into feed by *P. sajor-caju*. The lignin content decreased from 44.36 to 25.53% for sawdust and from 20 to 14.2% for CBP. The nitrogen vis-a-vis proteins were also increased significantly, i.e., 0.35 to 1.25% in sawdust and 1.52 to 2.48% in CBP.

Similarly various *Pleurotus* sp. have been tested for bioconversion of citrus bagasse and rice straw into animal feed. Out of four species of *Pleurotus*, *P. ostreatus* 814 was found to be efficient in improving the protein level (Albores et al. 2006), and a scale-up process, using rice straw bales in the open, is under progress. Thus, globally there are constant efforts to develop fastest method, where lignin is preferentially degraded without touching much of the cellulose. Such efficient method eventually will boost biopulping as well as animal feed production technologies. Li et al. (2008) successfully screened *Fusarium concolor* as an efficient organism for selective lignin degradation, and it was found to degrade 13% of lignin with only 7% loss of holocellulose after 5 days. In another distinct attempt, our group characterized the *Pleurotus ostreatus*- and *Trametes versicolor*-fermented wheat straw using in vitro gas production for its metabolizable energy and digestibility (Shrivastava et al. 2011). The 10th day was found to be best to attain the maximum efficiency of SSF based on the nutritive value and selective lignin degradation.

In a very recent report Sharma and Arora (2010a) have optimized conditions for production of lignocellulolytic enzymes by *Phlebia floridensis* during solid-state fermentation of wheat straw along with enhancement of in vitro digestibility, and response surface methodology (RSM)-based experiment was performed. Effect of moisture content, inorganic nitrogen source (NH_4Cl) and malt extract on lignocellulolytic enzymes, changes in chemical constituents, and digestibility of wheat straw was evaluated. With increase in moisture content, laccase production was found to increase up to 34-fold, while Manganese peroxidase was optimally produced in the presence of almost equal amount (50–55 mg g^{-1} of WS) of NH_4Cl and malt extract. These supplements also significantly ($p<0.05$) enhanced the production of CMCase and xylanase. In vitro digestibility was increased by almost 50% with a loss of 27.6 and 14.6% in lignin and total organic matter, respectively. The findings revealed that *P. floridensis* was an efficient organism for lignocellulolytic enzymes production and simultaneous enhancement in in vitro digestibility of wheat straw. However, despite having such kind of studies, complete optimization of the process is yet to be done and is in progress in our laboratory. In another recent report, Arora et al. (2011) tested various fungi for bioconversion process, and among them *Phlebia brevispora* degraded maximum lignin (30%) and enhanced digestibility from 172 to 287 g kg^{-1} along with an increase in antioxidant property of fermented straw.

Only a few processes reached pilot plant scale and none so far to commercial scale. Among them, pilot scale semisolid fermentation of ryegrass straw was carried out in a pilot plant at 100-kg scale using *Candida utilis,* and the fermentation was found to increase protein content, crude fat, and in vitro rumen digestibility (up to 50%) (Grant et al. 1978). For the large-scale

animal feed production in a reactor, Kumar and Gomes (2008) elaborated the chronology of reactors' design from lab to pilot scale for the bioconversion of wheat straw into animal feed using *P. chrysosporium*. Performances of designs were compared between horizontal, fluidized, and vertical based on engineering and bioconversion parameters. During scale-up of solid-state bioconversion process, poor conductivity of lignocellulosic substrate, the metabolic heat generated causing spatial temperature gradient, and mass transfer were figured out as major problems. The staged vertical reactor (design V-G2b) was studied in detail. The response surface generated from the data showed that the maximum value of the desirability coefficient obtained was 0.752 for an inoculum size of 0.35 g/100 g of dry wheat straw, a wheat straw loading of 1.5 kg per stage, and an air flow rate of 15.0 L min^{-1}. The lignin and cellulose degradation achieved was 27 and 29%, respectively. No single reactor design can solve all the problems faced in solid-state bioconversion processes and can provide solutions only to particular problems. Finally, a vertical deep bed reactor was designed with special impellers that ensured the circulation of the solid substrate. The average mixing characteristic time is 3 min. The 1,200-L reactor, a steam generator, sterile air supply system, and seed culture tank together constituted a complete design, and it is capable of stand-alone operation.

Alternatively, various other strategies, involving single or simultaneous organisms and sequential consortia, have been tried (Adamovic et al. 1998; Nigam 1998). Currently, *C. utilis* and *P. ostreatus* have been evaluated for conversion of apple pomace into nutritionally enriched and digestible animal feed, either individually or in sequential order (Villas-Boas et al. 2003). The increase in crude protein obtained almost 100%, which was accompanied by 8.2% rise in the digestibility when apple pomace fermented with *C. utilis* alone. While the crude protein increased by 10.5% (wt dry wt^{-1} of the sample), when substrate was fermented in sequential manner. However, no lignin degradation was observed. More recently, Chi et al. (2007) have cocultured various white-rot fungi to study the degradation of aspen wood. They have reported first time that coculture of *P. ostreatus* with *C. subvermispora* significantly stimulated more laccase production and had shown that cocultivation of wood increases wood degradation and alters the lignin degradation pattern toward a more recalcitrant part of lignin; however, no significant increase in total lignin removal was obtained

Based on the literature, lignin degradation and mineralization largely appears to be exclusive domain of white-rot fungi. This selecting ability of white-rot fungi to degrade lignin preferably from agricultural crop waste and agro-industrial waste could be exploited for their conversion into nutritional-rich animal feed. Based on the large-scale screening program carried to select better delignifier, the most selective fungi were *D. squalens*, *Phlebia* sp., *Ceriporiopsis subvermispora,* and *C. rivulosa* (Otjen et al. 1988; Akhtar et al. 1997; Hakala et al. 2004, 2005; Okano et al. 2009). However, the mechanism of selective degradation of lignin has not been elucidated. Thus, if selective lignin-degrading white-rot fungi are grown under optimized conditions and by manipulating the culture condition or lignin-degrading genes using modern molecular biological tools, it would be possible to tackle the feed problem round the corner of the world.

Molecular Biology of Lignin Degradation

Lignin depolymerization system constitutes multiple isozymes and corresponding genes of laccase, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and versatile peroxidases (Have and Teunissen 2001). As mentioned earlier, robust investigations have been made on fungal system of lignin degradation, viz., biochemical and biophysical characterization of enzymes and their production. It has been observed in literature that the production of these enzymes from various microbial sources is too low for commercial applications. To overcome this hurdle, molecular cloning and characterization of fungal laccases as well as improvement of the expression level using homologous as well as

heterologous expression systems have been carried out (Kunamneni et al. 2008).

Research has been carried out concerning the molecular genetics of lignin degradation by white-rot fungi, particularly with *P. chrysosporium*. Tien and Tu (1987) were the first to report the cloning of the cDNA-encoding lignin peroxidase H8. Since then much reports have been published about the number, structure, and organization of the *P. chrysosporium* peroxidase genes. However, there is still uncertainty about the exact number and structure of LiP genes (Highley and Dashek 1998). The LiP genes in *P. chrysosporium* have been reported to vary from 5 to 15 (Gaskell et al. 1994). An RFLP-based genetic map localized LiP genes of *P. chrysosporium* isolate ME446 to two linkage groups (Raeder et al. 1989). Following chromosome separation by clamped homogenous electrical field (CHEF) electrophoresis, five LiP genes were assigned to a single chromosome. In agreement with the RFLP map, another LiP clone (GLG4) was assigned to the same chromosome as a cbhl cluster (Gaskell et al. 1994). Cullen and Kersten (1992) have shown that at least one MnP gene resides on the same chromosome as do five LiP. Thereafter, Stewart and his team (1996) reported the successful expression of MnP in *Aspergillus oryzae*. Moreover, occurrence of multiple LiPs has been reported in *Bjerkandera adusta*, *C. versicolor,* and *Fomes lignosus* (Huoponen et al. 1990).

Several LiP genes have been characterized from other fungal species, including four *T. versicolor* clones LPGI (Jonsson and Nyman 1992), LPGII (Jonsson and Nyman 1994), VLGI (Black and Reddy 1991), LPGVI, *Bjerkandera adusta* clone LPO-1, and *Phlebia radiata lpg3* (Saloheimo et al. 1989). On the basis of Southern blot hybridization to the *P. chrysosporium* genes, LiP-like sequences also appear to be present in the genomes of *Fomes lignosus* (Huoponen et al. 1990), *P. brevispora,* and *C. subvermispora* (Rajakumar et al. 1996). PCR amplification of LiP-like sequences also suggests the existence of functional LiP genes in *C. subvermispora* and *P. sordida,* species that lack detectable lignin peroxidase activity (Rajakumar et al. 1996). Sugiura et al. (2009) have successfully cloned LiP genes from *Phanerochaete sordida* and their homologous expression system constructed. They used promoter of glyceraldehyde-3-phosphate dehydrogenase to drive the expression of cloned LiP genes.

Similar to LiP, the multiple isozymes of MnP have been reported from *P. chrysosporium.* Alic et al. (1997) have reported four MnP genes from this fungus. There are also numerous reports on the MnPs of white-rot fungi such as *P. ostreatus* (Kamitsuji et al. 2004), *T. versicolor* (Johansson et al. 2002), and others (Manubens et al. 2003; Hakala et al. 2006). MnPs in white-rot fungi have conserved amino acid sequences for metal binding regions, and the nucleotide sequences in those regions can be used as the PCR primers for gene cloning (Kim et al. 2005). More recently, MnP-encoding genes have been successfully cloned and characterized (Yeo et al. 2007; Nagai et al. 2007; Sakamoto et al. 2009).

Multiple sequence alignment reveals features that help to distinguish MnP and LiP; putative Mn^{2+}-biding residues have been identified in MnP genes (Sundaramoorthy et al. 1994). Excluding *T. versicolor,* the MnP gene can be distinguished by the presence of 7–11 amino acid surface loop (Sundaramoorthy et al. 1994) and an extended terminus. The loop contains a fifth disulfide bond, which is not found in LiP genes. Heterologous gene expression of MnP and LiP genes has been reviewed by Pease and Tien (1992). Baculovirus system has been used to produce active recombinant MnP isozyme H4. The recently sequenced enzymes MnPL1 and MnPL2 from cultures of *P. eryngii* exhibit high sequence and structural similarities with LiP from *P. chrysosporium* (Moreira et al. 2005).

Discovery of the ligninolytic peroxidases leads to the isolation of the responsible genes from various white-rot fungi (Gold and Alic 1993; Asada et al. 1995; Johansson and Nyman 1996; Kimura et al. 1990; Lobos et al. 1998). Several regulatory elements have been described in the promoter regions of LiP and MnP. Promoter regions of LiP or MnP genes studied so far contain cAMP response elements (CRE) to notice starvation. Aside from starvation, the presence of Mn^{2+} is essential for MnP gene expression in

P. chrysosporium and *Dichomitus squalens* (Périé and Gold 1991; Brown et al. 1991). The observation is in agreement with the general finding that elevated manganese levels are beneficial for the production of MnP in many white-rot fungi. Fifty-eight putative metal response elements (MRE) were found in *P. chrysosporium* which are similar to a gene that encodes for a mouse metalloprotein (Gold and Alic 1993; Alic et al. 1997). There are two different laccases which have been distinguished on the basis of gene expression: constitutive and inducible (Bollag and Leonowicz 1984; Yaver et al. 1996). Induction of laccase has been observed at the level of transcription and translation upon addition of copper, xylidine, veratric acid, etc. (Bollag and Leonowicz 1984; Palmieri et al. 2000).

Genome sequence of the most intensively studied white rot, *P. chrysosporium,* is now unraveled by Martinez et al. (2004). Since LiP and MnP require extracellular H_2O_2 for their in vivo catalytic activity, likely source for this is the six sequences of copper radical oxidase (cro1 through cro6), glyoxal oxidase GLOX, and extracellular FAD-dependent oxidases (Kersten and Kirk 1987; Kersten et al. 1990; Kersten and Cullen 2007). Genes encoding FAD oxidases in related white-rot fungi include aryl alcohol oxidases (AAO) from *P. eryngii* and pyranose oxidase from *C. versicolor*. Four distinct AAO-like sequences, a pyranose oxidase-like sequence, and a glucose oxidase-like sequence have been identified in the genome data. The precise roles and interactions of these genes in lignin degradation still remain to be determined (Ander and Maezullo 1997). Production of H_2O_2 by AAO through aromatic-aldehyde redox cycling provides a continuous source of oxidative power for lignin degradation. H_2O_2 participates in reactions catalyzed by ligninolytic peroxidases and acts as a precursor of hydroxyl-free radical ($OH^{\bullet}$).

In addition to H_2O_2 production, AAO also prevents the repolymerization of products released from enzymatic degradation of lignin. Genome searches revealed no conventional laccases in *P. chrysosporium*. Instead, four multicopper oxidase (MCO) sequences are found clustered within a 25-kb segment on scaffold 56. Thus, it appears that *P. chrysosporium* does not have the capacity to produce laccases although distantly related multicopper oxidases may have a role in extracellular oxidations. In addition to lignin, *P. chrysosporium* completely degrades all major components of plant cell walls including cellulose and hemicellulose. The genome harbors the genetic information to encode more than 240 putative carbohydrate-active enzymes (Henrissat 1991) (http://afmb.cnrs-mrs.fr/CAZY/) including 166 glycoside hydrolases, 14 carbohydrate esterases, and 57 glycosyltransferases, comprising at least 69 distinct families.

The progress in research work on the transcriptional regulation of peroxidases has been hampered by difficulties in distinguishing closely related genes (Cullen and Kersten 1992). However, it is obvious that LiP genes are transcriptionally regulated and that expression of MnP genes is Mn^{2+} dependent (Brown et al. 1991), although the specificity of the transcripts observed on Northern blots is questionable (Cullen and Kersten 1992).

Versatile peroxidases (VPs) are heme enzymes that combine catalytic properties of lignin peroxidases and manganese peroxidases, being able to oxidize Mn (II) as well as phenolic and nonphenolic aromatic compounds in the absence of mediators. The catalytic process, initiated by hydrogen peroxide, is the same of classical peroxidases with the involvement of two oxidizing equivalents and the formation of the so-called Compound I. This latter state contains an oxoferryl center and an organic cation radical which can be located either on the porphyrin ring or on a protein residue (Pogni et al. 2006).

Two genes encoding VP isoenzymes VPL and VPS1, expressed in liquid and solid-state fermentation cultures, respectively, have been cloned from *P. eryngii* (Ruiz-Dueñas et al. 1999). The deduced amino acid sequences for both isoenzymes were used to build molecular models by homology modeling, taking advantage from sequence identity with *P. chrysosporium* LiP and MnP and *Coprinopsis cinerea* peroxidase (CIP). The structure of the peroxidase from *C. cinereus* (CiP) has been determined in three different space groups and crystalline environments

(Houborg et al. 2003). By combining a homologous recombinant gene expression system and optimization of the culture conditions, hyper overproduction of *Pleurotus ostreatus* versatile peroxidase MnP2 has been achieved (Tsukihara et al. 2006). They have reported 7,300 U L^{-1} of MnP from recombinant strain TM2-18, more than a 30-fold overproduction as compared to the previous reports. Ruiz-Duenas et al. (2001) have reported two versatile peroxidases from *Pleurotus* and *Bjerkandera* spp., which have been cloned, sequenced, and characterized. They have reported to show their high affinity for Mn^{2+}, hydroquinones, and dyes and also oxidize veratryl alcohol, dimethoxybenzene, and lignin dimers. Huang et al. (2009) have cloned and characterized a novel ligninolytic peroxidase gene (ACLnP) from a poroid brown-rot fungus, *Antrodia cinnamomea*. ACLnP that was cloned into vector pQE31 and successfully expressed in *E. coli* strain M15 under the control of the T5 promoter produced a non-glycosylated protein of about 38 kDa, p*I* 5.42.

Literature survey indicates that various strategies have been used to isolate and clone the laccase coding sequences from fungal wild type. The laccase genes have been isolated, cloned, and characterized based on the sequence information of purified protein of *Coriolus hirsutus* (Kojima et al. 1990), *P. ostreatus* (Giardina et al. 1995; Giardina et al. 1999), *T. versicolor* (Jonsson et al. 1995), and *Neurospora crassa* (Germann et al. 1988) by screening of genomic or cDNA libraries using probes designed on the basis of partial amino acid sequence of fungal laccases. To clone laccase genes, similar PCR methods based on conserved Cu-binding regions have also been used (D'Souza et al. 1996; Temp et al. 1999; Ducros et al. 1998; Hoshida et al. 2001). In a novel approach, Joo et al. (2008) have reported to isolate the laccase-specific genomic sequence applying inverse PCR and subsequently, cloned and expressed the laccase in *Pichia pastoris*. The cDNA corresponding fungal laccase comprise of 1,554–1,563 nucleotides encoding a protein of 518–520 amino acids with about 21–23 signal peptide. The gDNA of laccase reported to date has variable number of introns ranging from 10 to 12 interrupting the coding sequence.

Homologous expression systems for extracellular proteins of Basidiomycetes have been reported for several enzymes including laccase (Mayfield et al. 1994; Sollewijn Gelpke et al. 1999; Irie et al. 2001; Ma et al. 2003; Kajita et al. 2004). Similarly, the heterologous expression of fungal genes for laccases has been achieved in filamentous fungi (Saloheimo et al. 1991; Yaver et al. 1996; Cassland and Jönssson 1999; Liu et al. 2003; Record et al. 2002; Kiiskinen et al. 2004; Rodríguez et al. 2008). In this regard, several approaches have also been described in order to successfully express basidiomycetous laccases using yeast (Jönsson et al. 1997; Hoshida et al. 2001; Piscitelli et al. 2005; Jolivalt et al. 2005; Guo et al. 2005; Bohlin et al. 2006; Faraco et al. 2008; Kim et al. 2010; López et al. 2010; Huang et al. 2011). Survey of literature clearly indicates that both homo- and heterologous expression systems have been used in laccase cloning and expression.

Kilaru et al. (2006) reported the homologous expression of *C. cinerea* laccase gene under non-inductive conditions using various homologous and heterologous promoters. They observed that irrespective of promoter used, addition of Cu to the medium increased enzymatic activity by 10–50-fold. However, promoter efficiency for heterologous expression needs extensive evaluation. O'Callaghan et al. (2002) studied optimization of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris* under shake flask culture. They described the development of a medium that allows convenient pH control of *P. pastoris* without the need for continuous neutralization. Quite recently, López et al. (2010) studied growth kinetics of *Pichia pastoris* and heterologous expression of *Trametes versicolor* laccase under solid-state (SSF) and submerged fermentations (SmF). For the first time, they used polyurethane foam (PUF) for solid-state yeast cultures and observed that this system may be a promising and simple way to produce heterologous proteins with *P. pastoris*. The results showed the strong inhibition of laccase production in the

SmF experiments, compared with the high laccase titer in the SSF experiments. They observed that oxygen mass transfer is more efficient in SSF which is related to the higher area/volume ratio compared with SmF.

We have been working on molecular genetics of the lignin degradation in our laboratory and have reported the efficient and convenient *Agrobacterium*-mediated gene transformation system in fungi (Sharma et al. 2006). We successfully delivered the T-DNA carrying the genes coding for β-glucuronidase (uidA), green fluorescent protein (gfp), and hygromycin phosphotransferase (hpt) to the nuclear genome of lignin-degrading white-rot fungi such as *Phanerochaete chrysosporium, Ganoderma* sp. RCKK-02, *Pycnoporus cinnabarinus, Crinipellis* sp. RCK-1, *Pleurotus sajor-caju,* and fungal isolate BHR-UDSC. This methodology will provide a rapid and reproducible transformation method without external addition of acetosyringone, which could be useful for improving white-rot fungi for their various biotechnological applications (Sharma and Kuhad 2010).

Conclusion

The mechanism of lignin degradation by fungi has been understood to a larger extent; however, knowledge about the mechanism that promotes selective delignification of plant cell wall is limited. This necessitates the need to understand the culture conditions promoting delignification, such as effect of (1) pure oxygen than in presence of air, (2) manganese in the culture medium, (3) nitrogen and its concentration, and (4) certain inducers. Lignocellulose bioconversion by SSF will have an important role in future biotechnologies, and animal feed production will be one of them, mainly because of its favorable economy, larger availability, and ease of on-site operation in agricultural facilities. For faster conversion of lignocellulosic material into digestible and protein-rich animal feed, there is need to design an appropriate bioreactor to run solid-state fermentation by selective lignin-degrading fungi. There is further need to improve the white-rot fungi for their lignin-degrading ability, which could be achieved following rDNA technology.

Despite the good level on knowledge of biochemistry and genetics of microbial degradation, still there are limitations to achieve complete exploitation of plant-based resources to value-added products, especially animal feed. Several aspects of lignin degradation still remain unsolved. Among them is the knowledge about the mechanism of lignin degradation occurring in the environment and to what extent there are different microorganism, especially white-rot fungi, involved in this process. Moreover, the production level from the best known culturable white-rot fungi is low, which is the matter of concern in improving delignification of plant material and eventually economization of the bioconversion process. Thus, to overcome the existing problems, the effort should be to analyze total DNA from various environments and to hunt for the robust ligninolytic gene(s) or biocatalyst(s). If we succeed getting a robust system, which fastens lignin degradation in lesser duration, the problem of animal feed can be solved to a larger extent.

References

Abdullah N, Khan AD, Ejaz N (2004) Influence of nutrients carbon and nitrogen supplementation on biodegradation of wheat straw by *Trametes versicolor*. Mycol Appl Int 16:7–12

Adamovic M, Grubic G, Milenkovic I, Jovanovic R, Protic R, Sretenovic L, Stoicevic L (1998) The biodegradation of wheat straw by *Pleurotus ostreatus* mushrooms and its use in cattle feeding. Anim Feed Sci Technol 71:357–362

Adhi TP, Korus RA, Crawford DL (1989) Production of major extracellular enzymes during lignocellulose degradation by two *Streptomyces* in agitated submerged culture. Appl Environ Microbiol 55: 1165–1168

Aggelis G, Ehaliotis C, Nerud F, Stoychev I, Lyberatos G, Zervakis G (2002) Evaluation of white-rot fungi for detoxification and decolorization of effluents from the green olive debittering process. Appl Microbiol Biotechnol 59:353–360

Agosin E, Monties B, Odier E (1985) Structural changes in wheat straw components during decay by lignin-degrading white-rot fungi in relation to improvement of digestibility for ruminants. J Sci Food Agric 36:925–935

Aguirre F, Maldonado O, Rolz C, Menchu JF, Espinosa R, de Cabrera S (1976) Protein from waste: growing fungi on coffee waste. Chem Technol 6:636–642

Ahmad M, Taylor CR, Pink D, Burton K, Eastwood D, Bending GR, Bugg TDH (2010) Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders. Mol Biosyst 6:815–821

Akhtar M, Blanchette RA, Kirk TK (1997) Fungal delignification and biomechanical pulping of wood. In: Scheper T (ed) Advances in biochemical engineering/biotechnology, vol 57. Springer, Berlin/Heidelberg, pp 159–195

Akin DE (1993a) In: Shimada K, Ohmiya K, Kobayashi Y, Hoshino S, Sakka K, Karita S (eds) Genetics biochemistry and ecology of lignocellulose degradation. UNI, Tokyo, p 95

Akin DE, Sethuraman A, Morrison WH III, Martin SA, Eriksson K-EL (1993b) Microbial delignification with white-rot fungi improves forage digestibility. Appl Environ Microbiol 61:1591–1598

Akinyele BJ, Olaniyi OO, Arotupin DJ (2011) Bioconversion of selected agricultural wastes and associated enzymes by *Volvariella volvacea*: an edible mushroom. Res J Microbiol 6:63–70

Albores S, Pinazzola MJ, Soubes M, Cedeiras MP (2006) Biodegradation of agroindustrial wastes by *Pleurotus* sp. for its use as ruminant feed. Electron J Biotechnol 9:215–220

Alexandre G, Zhulin IB (2000) Laccases are widespread in bacteria. Trends Biotechnol 18:41–42

Alic M, Akileswaran L, Gold MH (1997) Characterization of the gene encoding manganese peroxidase isozyme 3 from *Phanerochaete chrysosporium*. Biochim Biophys Acta 1338:1–7

Amitai G, Adani R, Sod-Moriah G, Rabinovitz I, Vincze A, Leader H, Chefetz B, Leibovitz-Persky L, Friesem D, Hadar Y (1998) Oxidative biodegradation of phosphorothiolates by fungal laccase. FEBS Lett 438:195–200

Ander P, Maezullo L (1997) Sugar oxidoreductases and veratryl alcohol oxidases as related to lignin degradation. J Biotechnol 53:115–131

Archana PI, Mahadevan A (2002) Lignin degradation by bacteria. Prog Ind Microbiol 36:311–330

Arias ME, Arenas M, Rodriguez J, Soliveri J, Ball AS, Hernandez M (2003) Kraft pulp biobleaching and mediated oxidation of a non phenolic substrate by laccase from Streptomyces cyaneus CECT 3335. Appl Environ Microbiol 69:1953–1958

Arora DS, Gill PK (2001) Effects of various media and supplements on laccase production by some white rot fungi. Bioresour Technol 77:89–91

Arora DS, Rampal P (2002) Laccase production by some *Phlebia* species. J Basic Microbiol 42:295–301

Arora DS, Chander M, Gill PK (2002) Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. Int Biodeter Biodegr 50:115–120

Arora DS, Sharma RK, Chandra P (2011) Biodelignification of wheat straw and its effect on in vitro digestibility and antioxidant properties. Int Biodeter Biodegr 65:352–358

Asada Y, Watanabe A, Irie T, Nakayama T, Kuwahara M (1995) Structures of genomic and complementary DNAs coding for *Pleurotus ostreatus* manganese (II) peroxidase. Biochim Biophys Acta 1251:205–209

Ball A, Betts W, McCarthy A (1989) Degradation of lignin-related compounds by Actinomycetes. Appl Environ Microbiol 55:1642–1644

Bals B, Murnen H, Allen M, Dale B (2010) Ammonia fiber expansion (AFEX) treatment of eleven different forages: improvements to fiber digestibility in vitro. Anim Feed Sci Technol 155:147–155

Bao WJ, Usha SN, Renganathan V (1993) Purification and characterization of cellobiose dehydrogenase, a novel extracellular hemoflavoenzyme from the white-rot fungus Phanerochaete chrysosporium. Arch Biochem Biophys 300:705–713

Barr DP, Aust SD (1994) Mechanisms white-rot fungi use to degrade pollutants. Environ Sci Technol 28:78–87

Basu S, Gaur R, Gomes J, Sreekrishnan TR, Bisaria VS (2002) Effect of seed culture on solid state bioconversion of wheat straw by *Phanerochaete chrysosporium* for animal feed production. J Biosci Bioeng 1:25–30

Belewu MA (2006) Conversion of masonia tree sawdust and cotton plant by product into feed by white rot fungus (*Pleurotus sajor caju*). Afr J Biotechnol 5:503–504

Belyaeva ON, Haynes RJ (2009) Chemical, microbial and physical properties of manufactured soils produced by co-composting municipal green waste with coal fly ash. Bioresour Technol 100:5203–5209

Bhatnagar A, Kumar S, Gomes J (2008) Operating conditions of a 200 l staged vertical reactor for bioconversion of wheat straw by *Phanerochaete chrysosporium*. Bioresour Technol 99:6917–6927

Black AK, Reddy CA (1991) Cloning and characterization of a lignin peroxidase gene from the white-rot fungus *Trametes versicolor*. Biochem Biophys Res Commun 179:428–435

Blanchette RA (1995) Degradation of lignocellulose complex in wood. Can J Bot 73:S999–S1010

Blanchette RA, Abad AR, Farrell RL, Leathers TD (1989) Detection of lignin peroxidase and xylanase by immunocytochemical labeling in wood decayed by basidiomycetes. Appl Environ Microbiol 55:1457–1465

Blanchette RA, Burnes TA, Eerdmans MM, Akhtar M (1992) Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora* for use in biological pulping processes. Holzforschung 46:109–115

Boer CG, Obici L, de Souza CGM, Peralta RM (2006) Purification and some properties of Mn peroxidase from *Lentinula edodes*. Process Biochem 41:1203–1207

Bohlin C, Jönsson LJ, Roth R, Vanzyl WH (2006) Heterologous expression of *Trametes versicolor* laccase in *Pichia pastoris* and *Aspergillus niger*. Appl Biochem Biotechnol 129–132:195–214

Bollag JM, Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48:849–854

Bonnarme P, Jeffries TW (1990) Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl Environ Microbiol 56:210–217

Bonnen AM, Anton LH, Orth AB (1994) Lignin-degrading enzymes of the commercial button mushroom *Agaricus bisporus*. Appl Environ Microbiol 60:960–965

Bonugli-Santos RC, Durrant LR, Sette LD (2010) Production of laccase, manganese peroxidase and lignin peroxidase by Brazilian-derived fungi. Enzyme Microb Technol 46:32–37

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102

Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Bornemann S (1996) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63:4627–4632

Breccia JD, Bettucci L, Sineriz F (1997) Degradation of sugarcane bagasse by several white-rot fungi. Acta Biotechnol 17:177–184

Brown JA, Alic M, Gold MH (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. J Bacteriol 173: 4101–4106

Brune A (2007) Woodworker's digest. Nature 450: 487–488

Bugg TDH, Ahmad M, Hardiman EM, Singh R (2011) The emerging role for bacteria in lignin degradation and bio-product formation. Curr Opin Biotechnol 22:394–400

Buswell JA, Odier E (1987) Lignin biodegradation. CRC Crit Rev Biotechnol 6:1–60

Call HP, Mucke I (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator systems (lignozyme(R)-process). J Biotechnol 53:163–202

Camarero S, Sarkar S, Ruiz-Duenas FJ, Martinez MJ, Martinez AT (1999) Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. J Biol Chem 274:10324–10330

Cambria MT, Cambria A, Ragusa S, Rizzarelli E (2000) Production, purification, and properties of an extracellular laccase from *Rigidoporus lignosus*. Protein Expr Purif 18:141–147

Capeleri M, Zadrazil F (1997) Lignin degradation and in vitro digestibility of wheat straw treated with Brazilian tropical species of white rot fungi. Folia Microbiol 42:481–487

Cassland P, Jönssson LJ (1999) Characterization of a gene encoding Trametes versicolor laccase A and improved heterologous expression in *Saccharomyces cerevisiae* by decreased cultivation temperature. Appl Microbiol Biotechnol 52:393–400

Chagas EP, Durrant LR (2001) Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sajorcaju*. Enzyme Microbiol Technol 29:473–477

Chahal DS, Moo-Young M, Dhillon GS (1979) Bioconversion of wheat straw and wheat straw components into single-cell protein. Can J Microbiol 25:793–797

Chandra R, Raj A, Purohit HJ, Kapley A (2007) Characterization and optimization of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. Chemosphere 67:839–846

Chen J, Fales SL, Varga GA, Royse DJ (1995) Biodegradation of cell wall components of maize stover colonized by white-rot fungi and resulting impact on in vitro digestibility. J Sci Food Agric 68:91–98

Chen S, Ge W, Buswell JA (2004) Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*. Europian. J Biochem 271:318–328

Chesson A (1981) Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. J Sci Food Agric 32:745–758

Chi Y, Hatakka A, Maijala P (2007) Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin degrading enzymes. Int Biodeter Biodegr 59:32–39

Chivukula M, Spadaro JT, Renganathan V (1995) Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. Biochemistry 34:7765–7772

Choi GH, Larson TG, Nuss DL (1992) Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. Mol Plant Microbe Interact 5:119–128

Claus H (2003) Laccases and their occurrence in prokaryotes. Arch Microbiol 179:145–150

Claus H, Decker H (2006) Bacterial tyrosinases. Syst Appl Microbiol 29:3–14

Clutterbuck AJ (1972) Absence of laccase from yellow-spored mutants of Aspergillus nidulans. J Gen Appl Microbiol 70:423–435

Couto SR, Herrera JLT (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24:500–513

Couto SR, Gundin M, Lorenzo M, Sanroman MN (2002) Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid-state conditions. Process Biochem 38:249–255

Cowling EB (1961) Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. US Dep Agr Tech Bull 1258. US Department of Agriculture, Washington, DC, 79 p

Cui F, Dolphin D (1990) The role of manganese in model systems related to lignin biodegradation. Holzforschung 44:279–283

Cullen D, Kersten P (1992) Fungal enzymes for lignocellulose degradation. In: Kinghorn JR, Turner G (eds) JR applied molecular genetics of filamentous fungi. Blackie Academic and Professional (Chapman & Hall), Glasgow, pp 100–131

Cullen D, Kersten PJ (2004) Enzymology and molecular biology of lignin degradation. In: Brambl R, Marzulf GA (eds) The Mycota III: biochemistry and molecular biology. Springer, Berlin, pp 249–273

D'Souza TM, Boominathan K, Reddy CA (1996) Isolation of lactase gene-specific sequences from white rot and brown rot fungi by PCR. Appl Environ Microbiol 62:3739–3744

D'Souza TM, Merritt CS, Reddy CA (1999) Lignin-modifying enzymes of the white rot basidiomycete Ganoderma lucidum. Appl Environ Microbiol 65: 5307–5313

Daniel GF, Nilsson T, Singh AP (1987) Degradation of lignocellulosics by unique tunnel-forming bacteria. Can J Microbiol 33:943–948

Das N, Chakraborty TK, Mukherjee M (1999) Role of potato extract in extracellular laccase production of *Pleurotus florida*. J Basic Microbiol 39:299–303

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578–595

Dashtban M, Schraft H, Syed TA, Qin W (2010) Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 1:36–50

De Jong E, De Vries FP, Field JA, Van der Zwan RP, De Bont JAM (1992) Isolation and screening of basidiomycetes with high peroxidative activity. Mycol Res 96:1098–1104

Deacon JW (1997) Modern mycology, 3rd edn. Blackwell Scientific, Oxford

Deswal D, Khasa YP, Kuhad RC (2011) Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. Bioresour Technol 102:6065–6072

Dey S, Maiti TK, Bhattacharyya BC (1994) Production of some extracellular enzymes by a lignin peroxidase-producing brown-rot fungus, *Polyporus osteiformis*, and its comparative abilities for lignin degradation and dye decolorization. Appl Environ Microbiol 60:4216–4218

Dhawan S, Kuhad RC (2002) Effect of amino acids and vitamins on laccase production by the bird's nest fungus *Cyathus bulleri*. Bioresour Technol 84:35–38

Dhawan S, Lal R, Hanspal M, Kuhad RC (2004) Effect of antibiotics on growth and laccase production from *Cyathus bulleri* and *Pycnoporus cinnabarinus*. Bioresour Technol 96:1415–1418

Dix NJ, Webster J (1995) Fungal ecology. Chapman and Hall, London

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH, Davie GJ (1998) Crystal structure of the pe-2 Cu depleted lactase from *Coprinus cinereus* at 2.2× resolution. Nat Struct Biol 5:310–316

Duran N, Esposito E (2000) Potential application of oxidative enzymes and phenoloxidase like compounds in wastewater and soil treatment: a review. Appl Catal Environ 28:83–99

Edens WA, Goins TQ, Dooley D, Henson JM (1999) Purification and characterization of a secreted laccase of Gaeumannomyces graminis var. tritici. Appl Environ Microbiol 65:3071–3074

Eggert C, Temp U, Dean JFD, Eriksson K-EL (1996) A fungal metabolite mediates oxidation of non-phenolic lignin model compounds and synthetic lignin by laccase. FEBS Lett 391:144–148

Eggert C, Lafayette PR, Temp U, Eriksson KEL, Dean JFD (1998) Molecular analysis of a laccase gene from white rot fungus *Pycnoporus cinnabarinus*. Appl Environ Microbiol 64:3151–3157

Ek M, Eriksson K-E (1975) Conversion of cellulosic waste into protein. Appl Polym Symp 28:197–203

Ek M, Eriksson K-E-L (1980) Utilization of the white-rot fungus *Sporotrichum pulverulentum* for water purification and protein production on mixed lignocellulosic wastewaters. Biotechnol Bioeng 22:2273–2284

Elisashvili V, Kachlishvili E, Penninckx M (2008) Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot *basidiomycetes*. J Ind Microbiol Biotechnol 35:1531–1538

Endo K, Hayashi Y, Hibi T, Hosana K, Beppu T, Ueda K (2003a) Enzymological characterization of EpoA, a laccase like phenol oxidase produced by Streptomyces griseus. J Biochem 133:671–677

Endo K, Hosono K, Beppu T, Ueda K (2003b) A novel extracytoplasmic phenol oxidase of Streptomyces: its possible involvement in the onset of morphogenesis. Microbiology 148:1767–1776

Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin/Heidelberg/New York

Fahr K, Wetzstein H-G, Grey R, Schlosser D (1999) Degradation of 2,4-dichlorophenol and pentachlorophenol by two brown rot fungi. FEMS Microbiol Lett 175:127–132

Fakoussa RM, Hofrichter M (1999) Biotechnology and microbiology of coal degradation. Appl Microbiol Biotechnol 52:25–40

Faraco V, Ercole C, Festa G, Giardina P, Piscitelli A, Sannia G (2008) Heterologous expression of heterodimeric laccase from *Pleurotus ostreatus* in *Kluyveromyces lactis*. Appl Microbiol Biotechnol 77:1329–1335

Fenice M, Sermanni GG, Federici F, D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. J Biotechnol 100:77–85

Ferrer I, Esposito E, Duran N (1992) Lignin peroxidase from *Chrysonilia sitophila*: heat denaturation kinetics and pH stability. Enzyme Microb Technol 14:402–406

Flachowsky G, Kamra DN, Zadrazil F (1999) Cereal straws as animal feed-possibilities and limitations. J Appl Anim Res 16:105–118

Freeman IC, Nayar PG, Begley TP, Villafranca JJ (1993) Stoichiometry and spectroscopic identity of copper centers in phenoxazinone synthase: a new addition to the blue copper oxidase family. Biochemistry 32:4826–4830

Galkin S, Vares T, Kalsi M, Hatakka A (1998) Production of organic acids by different white-rot fungi as detected using capillary zone electrophoresis. Biotechnol Tech 12:267–271

Galliano H, Gas G, Seris JL, Boudet AM (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. Enzyme Microb Technol 13:478–482

Garg SK, Neelakantan S (1982) Studies on the properties of cellulase enzyme from Aspergillus terreus, GNI. Biotechnol Bioeng 24:737–742

Gaskell J, Stewart P, Kersten P, Covert S, Reiser J, Cullen D (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction: application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. Bio/technology 12:1372–1375

Gassara F, Brar SK, Tyagi RD, Verma M, Surampalli RY (2010) Screening of agro-industrial wastes to produce ligninolytic enzymes by *Phanerochaete chrysosporium*. Biochem Eng J 49:388–394

Geib SM, Filley TR, Hatcher PG, Hoover K, Carlson JE, del Mar Jimenez-Gasco M, Nakagawa-Izumi A, Sleighter RL, Tien M (2008) Lignin degradation in wood-feeding insects. Proc Natl Acad Sci USA 105:12932–12937

Gelpke MDS, Mayfield M, Cereghino GPL, Gold MH (1999) Homologous expression of recombinant lignin peroxidase in *Phanerochaete chrysosporium*. Appl Environ Microbiol 65:1670–1674

Germann UA, Millller G, Hunziker PE, Lerch K (1988) Characterization of two allelic forms of *Neurospora crassa* laccase: amino- and carboxyl-terminal processing precursor. J Biol Chem 263:885–896

Ghosh A, Frankland JC, Thurston CF, Robinson CH (2003) Enzyme production by *Mycena galopus* mycelium in artificial media and in *Picea sitchensis* F1 horizon needle litter. Mycol Res 107:996–1008

Gianfreda L, Sannino F, Filazzola MT, Leonowicz A (1998) Catalytic behavior and detoxifying ability of a laccase from the fungal strain Cerrena unicolor. J Mol Catal B Enzym 4:13–23

Giardina P, Cannio R, Martirani L, Marzullo L, Palmieri G, Sannia G (1995) Cloning and sequencing of a lactase gene from the lignin-degrading basidiomycete *Pleurotus ostreatus*. Appl Environ Microbiol 61:2408–2413

Giardina P, Palmieri G, Scaloni A, Fontanella B, Farazo V, Cennamo G, Sannia G (1999) Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. Biochem J 341:655–663

Gilbertson RL (1980) Wood rotting fungi of North America. Mycologica 72:1–49

Gill PK, Arora DS (2003) Effect of culture conditions on manganese peroxidase production and activity by some white rot fungi. J Ind Microbiol Biotechnol 30:28–33

Glenn JK, Gold MH (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch Biochem Biophys 242:329–341

Glenn JK, Morgan MA, Mayfield MB, Kuwahara M, Gold MH (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 114: 1077–1083

Gold MH, Alic M (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol Rev 57:605–622

Gold MH, Wariishi H, Valli K (1989) Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. In: Whitaker JR, Sonnet PE (eds) Biocatalysis in agricultural biotechnology, ACS Symp. Ser. No. 389. The American Chemical Society, Washington, DC, pp 128–140

Grant GA, Han YM, Anderson AW (1978) Pilot-scale semi-solid fermentation of straw. Appl Environ Microbiol 35:549–553

Grey R, Hofer C, Schlosser D (1998) Degradation of 2-chlorophenol and formation of 2-chloro-1,4-benzoquinone by mycelia and cell-free crude culture liquids of *Trametes versicolor* in relation to extracellular laccase activity. J Basic Microbiol 38:371–382

Gunther T, Sack U, Hofrichter M, Latz M (1998) Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductases. J Basic Microbiol 38: 113–122

Guo M, Lu F, Pu J, Bai D, Du L (2005) Molecular cloning of the cDNA encoding laccase from *Trametes versicolor* and heterologous expression in *Pichia methanolica*. Appl Microbiol Biotechnol 69:178–183

Gupta BN (1987) Report: Indo-Dutch project on bioconversion of crop residues. National Dairy Research Institute, Karnal, pp 1–66

Gupta R, Mehta G, Khasa YP, Kuhad RC (2011) Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics. Biodegradation 22:797–804

Hakala TK, Maijala P, Konn J, Hatakka A (2004) Evaluation of novel wood rotting polypores and corticoid fungi for the decay and biopulping of Norway spruce(*Picea abies*) wood. Enzyme Microb Technol 34:255–263

Hakala TK, Lundell T, Galkin S, Maijala P, Kalkkinen N, Hatakka A (2005) Manganese peroxidases, laccase and oxalic acid from the selective white rot fungus *Physisporinus rivulosus* grown on spruce wood chips. Enzyme Microb Technol 36:461–468

Hakala T, Hilden K, Maijala P, Olsson C, Hadakka A (2006) Differential regulation of manganese peroxidases and characterization of two variable MnP encoding genes in the white-rot fungus *Physisporinus rivulosus*. Appl Microbiol Biotechnol 73:839–849

Hamman OB, de la Rubia T, Martinez J (1999) The effect of manganese on the production of *Phanerochaete flavido-alba* ligninolytic peroxidases in nitrogen limited cultures. FEMS Microbiol Lett 177:137–142

Han JR, An CH, Yuan JM (2005) Solid-state fermentation of cornmeal with the basidiomycete *Ganoderma lucidum* for degrading starch and upgrading nutritional value. J Appl Microbiol 99:910–915

Haselwandter K, Bobbleter O, Read DJ (1990) Degradation of 14C-labelled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. Arch Microbiol 153:352–354

Hatakka AI (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol Rev 13:125–135

Hatakka AI (2001) Biodegradation of lignin. In: Hofrichter M, Steinbüchel A (eds) Lignin, humic substances and coal, vol 1. Wiley-VCH, Weinheim, pp 129–180

Hatakka AI, Pirhonen TI (1985) Cultivation of wood rotting fungi on agricultural lignocellulosic materials for the production of crude protein. Agric Wastes 12:81–97

Hatakka AI, Kantelinen A, Tervilä-wilo A, Viikari L (1987) Production of ligninases by *Phlebia radiata* in agitated cultures. In: Odier E (ed) Lignin enzymic and microbial degradation, Symp Intern. INRA, Paris, pp 185–189

Hatvani N, Mecs I (2001) Production of laccase and manganese peroxidase by *Lentinus edodes* on malt-containing by-product of the brewing process. Process Biochem 37:491–496

Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U (1998) Transformation of industrial dyes by manganese peroxidases from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese independent reaction. Appl Environ Microbiol 64:2788–2793

Heinzkill M, Bech L, Halkier T, Schneider P, Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae*). Appl Environ Microbiol 64:1601–1606

Henriksson G, Johansson G, Pettersson G (2000) A critical review of cellobiose dehydrogenases. J Biotechnol 78:93–113

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Highley TL, Dashek WV (1998) Biotechnology in the study of brown- and white-rot decay. In: Bruce A, John W (eds) Forest products biotechnology. Taylor & Francis, London, pp 15–36

Higuchi T (1989) Mechanisms of lignin degradation by lignin peroxidase and laccase of white-rot fungi. In: Lewis NG, Paice MG (eds) Biogenesis and biodegradation of plant cell Polymers acs symposium series. American Chemical Society, Washington, DC, pp 482–502

Hofrichter M (2002) Review: Lignin conversion by manganese peroxidase (MnP). Enzyme Microb Technol 30:454–466

Hofrichter M, Fritsche W (1997) Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. Appl Microbiol Biotechnol 47:419–424

Hofrichter M, Scheibner K, Schneegass I, Fritsche W (1998) Enzymatic combustion of aromatic and aliphatic compounds by manganese peroxidase from *Nematoloma frowardii*. Appl Environ Microbiol 64:399–404

Hofrichter M, Scheibner K, Bublitz F, Schneegaß I, Ziegenhagen D, Martens R, Fritsche W (1999a) Depolymerization of straw lignin by manganese peroxidase from *Nematoloma frowardii* is accompanied by release of carbon dioxide. Holzforschung 53:161–166

Hofrichter M, Vares K, Scheibner K, Galkin S, Sipila J, Hatakka A (1999b) Mineralization and solubilization of synthetic lignin by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata*. J Biotechnol 67:217

Hofrichter M, Vares T, Kalsi M, Galkin S, Scheibner K, Fritsche W, Hataka A (1999c) Production of manganese peroxidase and organic acids and mineralisation of 14C-labelled lignin (14C-DHP) during solid-state fermentation of wheat straw with the white rot fungus *Nematoloma frowardii*. Appl Environ Microbiol 65:1864–1870

Hofrichter M, Lundell T, Hatakka A (2001) Conversion of milled pinewood by manganese peroxidase from *Phlebia radiata*. Appl Environ Microbiol 67:4588–4593

Hoshida H, Nakao M, Kanazawa H, Kubo K, Hakukawa T, Morimasa K, Akada R, Nishizawa Y (2001) Isolation of five laccase gene sequences from the white-rot fungus *Trametes sanguinea* by PCR, and cloning, characterization and expression of the laccase cDNA in yeasts. J Biosci Bioeng 92:372–380

Houborg K, Harris P, Poulsen JC, Schneider P, Svendsen A, Larsen S (2003) The structure of a mutant enzyme of *Coprinus cinereus* peroxidase provides an understanding of its increased thermostability. Acta Crystallogr D Biol Crystallogr 59:997–1003

Huang ST, Tzean SS, Tsai BY, Hsieh HJ (2009) Cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea*. Microbiology 155: 424–433

Huang DL, Zeng GM, Feng CL, Hu S, Lai C, Zhao MH, Su FF, Tang L, Liu HL (2010) Changes of microbial population structure related to lignin degradation during lignocellulosic waste composting. Bioresour Technol 101:4062–4067

Huang SJ, Liu ZM, Huang XL, Guo LQ, Lin J-F (2011) Molecular cloning and characterization of a novel laccase gene from a white-rot fungus *Polyporus grammocephalus* TR16 and expression in Pichia pastoris. Lett Appl Microbiol 52:290–297

Hungate RE (1966) The rumen and its microbes. Academic, New York

Huoponen K, Ollikka P, Kalin M, Walther I, Mantsala P, Reiser J (1990) Characterization of lignin peroxidase-encoding gene from lignin-degrading basidiomycetes. Gene 89:145–150

Hüttermann A, Milstein O, Nicklas B, Trojanowski J, Haars A, Kharazipour A (1989) Enzymatic modification of lignin for technical use. In: Glasser WG, Sarkanen S (eds) Lignin – properties and materials, ACS symposium series. ACS Publications, Washington, DC, pp 361–370

Iqbal M, Mercer DK, Miller PGG, McCarthy AJ (1994) Thermostable extracellular peroxidases from Streptomyces thermoviolaceus. Microbiology 140: 1457–1465

Irie T, Honda Y, Watanabe T, Kuwahara M (2001) Homologous expression of recombinant manganese peroxidase genes in ligninolytic fungus *Pleurotus ostreatus*. Appl Microbiol Biotechnol 55:566–570

Jalc D, Nerud F, Erbanova P, Siroka P (1996) Effect of white-rot basidiomycetes-treated wheat straw on rumen fermentation in artificial rumen. Reprod Nutr Dev 36:263–270

Jalc D, Siroka P, Ceresnakova Z (1997) Effect of six species of white-rot basidiomycetes on the chemical composition and rumen degradability of wheat straw. J Gen Appl Microbiol 43:133–137

Johansson T, Nyman PO (1996) A cluster of genes encoding major isozymes of lignin peroxidase, and manganese peroxidase from the white-rot fungus *Trametes versicolor*. Gene 170:31–38

Johansson T, Nymann P, Cullen D (2002) Differential regulation of mnp2, a new manganese peroxidase encoding gene from the lignolytic fungus *Trametes versicolor* PRL572. Appl Environ Microbiol 68:2077–2080

Jolivalt C, Madzak C, Brault A, Caminade E, Malosse C, Mougin C (2005) Expression of laccase IIIb from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica* for environmental applications. Appl Microbiol Biotechnol 66:450–456

Jonsson L, Nyman PO (1992) Characterization of a lignin peroxidase gene from the white rot fungus *Trametes versicolor*. Biochimie 74:177–182

Jonsson L, Nyman PO (1994) Tandem lignin peroxidase genes from the fungus *Trametes versicolor*. Biochim Biophys Acta 218:408–412

Jonsson L, SjSstrsm K, Hlggstriim I, Nyman PO (1995) Characterization of a laccase gene from the white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. Biochim Biophys Acta 1251:210–215

Jönsson L, Saloheimo M, Penttil M (1997) Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of lcc1 and expression in *Pichia pastoris*. Curr Genet 32:425–430

Joo SS, Ryu IW, Park JK (2008) Molecular cloning and expression of a laccase from *Ganoderma lucidum* and its antioxidative properties. Mol Cells 25: 112–118

Jung H-JG, Valdez FR, Abad AR, Blanchette RA, Hatfield RD (1992a) Effect of white rot basidiomycetes on chemical composition and in vitro digestibility of oat straw and alfalfa stems. J Anim Sci 70: 1928–1935

Jung H-JG, Valdez FR, Hatfield RD, Blanchette RA (1992b) Cell wall composition and degradability of forage stems following chemical and biological delignification. J Sci Food Agric 8:347–355

Kaal EEJ, Field JA, Joyce TW (1995) Increasing ligninolyic enzyme activities in several white-rot basidiomycetes by nitrogen-sufficient media. Bioresour Technol 53:133–139

Kahlon SS (1986) SCP production by *Chaetomium cellulolyticum* on treated waste cellulose. J Res Punjab Agric Univ 23:330–335

Kajita S, Sugawara S, Miyazaki Y, Nakamura M, Katayama Y, Shishido K, Iimura Y (2004) Overproduction of recombinant laccase using a homologous expression system in *Coriolus versicolor*. Appl Microbiol Biotechnol 66:194–199

Kakkar VK, Dhanda S (1998) Comparative evaluation of wheat and paddy straws for mushroom production and feeding residual straws to ruminants. Bioresour Technol 66:175–177

Kamitsuji H, Honda Y, Watanabe T, Kuwahara M (2004) Production and induction of manganese peroxidase isozymes in a white-rot fungus *Pleurotus ostreatus*. Appl Microbiol Biotechnol 65:287–294

Karunanandaa K, Varga GA (1996) Colonization of rice straw by white-rot fungi (*Cyathus stercoreus*): effect on ruminal fermentation pattern, nitrogen metabolism and fibre utilization during continuous culture. Anim Feed Sci Technol 61:1–16

Karunanandaa K, Fales SL, Varga GA, Akin DE, Rigsby LL, Royse DJ (1992) Chemical composition and biodegradability of crop residues colonized by white rot fungi. J Sci Food Agric 60:105–112

Kersten PJ, Cullen D (2007) Extracellular oxidative systems of the lignin-degrading Basidiomycete Phanerochaete chrysosporium. Fungal Genet Biol 44:77–87

Kersten PJ, Kirk TK (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J Bacteriol 169:2195–2201

Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar B, Kirk TK (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 268:475–480

Kiiskinen LL, Kruus K, Bailey M, Ylosmaki E, Siikaaho M, Saloheimo M (2004) Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology 150:3065–3074

Kilaru S, Hoegger PJ, Majcherczyk A, Burns C, Shishido K, Bailey A, Foster GD, Kües U (2006) Expression of laccase gene lcc1 in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Appl Microbiol Biotechnol 71:200–210

Kim Y, Yeo S, Kum J, Song H-G, Choi HT (2005) Cloning of a manganese peroxidase cDNA gene repressed by manganese in *Trametes versicolor*. J Microbiol 43:569–571

Kim J-M, Park S-M, Kim D-H (2010) Heterologous expression of a tannic acid-inducible laccase3 of *Cryphonectria parasitica* in *Saccharomyces cerevisiae*. BMC Biotechnol 10:18–27

Kimura Y, Asada Y, Kuwahara M (1990) Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl Microbiol Biotechnol 32:436–442

Kirk TK (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 268:475–480

Kirk TK, Cullen D (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. In: Young RA, Akhtar M (eds) Environmentally friendly technologies for the pulp and paper industry. Wiley, New York, pp 273–308

Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505

Kishi K, Wariishi H, Marquez L, Dunford HB, Gold MH (1994) Mechanism of manganese peroxidase compound II reduction. Effect of organic acid chelators and pH. Biochemistry 33:8694–8701

Klonowska A, Le Petit J, Tron T (2001) Enhancement of minor laccase production in the basidiomycete *Marasmius quercophilus* C30. FEMS Microbiol Lett 200:25–30

Kojima Y, Tsukuda Y, Kawai Y, Tsukamato A, Sugiura J, Sakaino M, Yukio K (1990) Cloning, sequence analysis and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J Biol Chem 260:15224–15230

Koroleva OV, Garilova VP, Stepanova EV, Lebedeva VI, Sverdlova NI, Landesman EO, Yavmetdinov IS, Yaropolov AI (2002) Production of lignin modifying enzymes by co-cultivated white-rot fungi *Cerrena maxima* and *Coriolus hirsutus* and characterization of laccase from *Cerrena maxima*. Enzyme Microb Technol 30:573–580

Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney CS (2003) Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS Microbiol Rev 27:663–693

Krcmar P, Ulrich R (1998) Degradation of polychlorinated biphenyl mixtures by the lignin-degrading fungus *Phanerochaete chrysosporium*. Folia Microbiol 43:79–84

Kristensen JB, Thygesen LG, Felby C, Jorgensen H, Elder T (2008) Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels 1:5

Kuhad RC, Singh A (1993) Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13:151–172

Kuhad RC, Singh A, Eriksson KE (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In: Eriksson KE (ed) Advances in biochemical engineering biotechnology. Springer, Berlin, pp 46–125

Kuhar S, Nair LM, Kuhad RC (2008) Pretreatment of lignocellulosic material with fungi capable of higher lignin degradation and lower carbohydrate degradation improves substrate acid hydrolysis and the eventual conversion to ethanol. Can J Microbiol 54:305–313

Kumar S, Gomes J (2008) Performance evaluation of reactors designed for bioconversion of wheat straw to animal feed. Anim Feed Sci Technol 144:149–166

Kumar AG, Sekaran G, Krishnamoorthy S (2006) Solid state fermentation of *Achras zapota* lignocellulose by *Phanerochaete chrysosporium*. Bioresour Technol 97:1521–1528

Kunamneni A, Camarero S, García-Burgos C, Plou FJ, Ballesteros A, Alcalde M (2008) Engineering and applications of fungal laccases for organic synthesis. Microb Cell Fact 7:32–49

Kurt S, Buyukalaca S (2010) Yield performances and changes in enzyme activities of *Pleurotus* spp. (*P. ostreatus* and *P. sajorcaju*) cultivated on different agricultural wastes. Bioresour Technol 101:3164–3169

Kuwahara M, Glenn JK, Morgan MA, Gold MH (1984) Separation and characterization of 2 extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett 169:247–250

Lackner R, Srebotnik E, Messner K (1991) Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of Phanerochaete chrysosporium. Biochem Biophys Res Commun 178: 1092–1098

Leatham G, Stahman MA (1981) Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J Gen Microbiol 125:147–157

Leonowicz A, Matuszewska A, Luterek J, Ziegenhagen D, Wojtas-Wasilewska M, Cho NS, Hofrichter M, Rogalski J (1999) Biodegradation of lignin by white rot fungi. Fungal Genet Biol 27:175–185

Levin L, Forchiassin F, Ramos AM (2001) Ligninolytic enzymes of the white-rot basidiomycete *Trametes trogii*. Acta Biotechnol 21:179–186

Levin L, Forchiassin F, Ramos AM (2002) Copper induction of lignin-modifying enzymes in the white-rot fungus *Trametes trogii*. Mycologia 94:377–383

Levy JF (1975) Bacteria associated with wood in ground contact. In: Liese W (ed) Biological transformation of wood by microorganisms. Springer, Berlin, pp 64–73

Li KC, Helm RF, Eriksson KEL (1998) Mechanistic studies of the oxidation of a non-phenolic lignin model compound by the laccase/1-hydroxybenzotriazole redox system. Biotechnol Appl Biochem 27:239–243

Li L, Li XZ, Tang WZ, Zhao J, Qu Y-B (2008) Screening of a fungus capable of powerful and selective delignification on wheat straw. Lett Appl Microbiol 47:415–420

Li J, Yuan H, Yang J (2009) Bacteria and lignin degradation. Front Biol China 4:29–38

Liese W (1970) Ultrastructural aspects of woody tissue disintegration. Annu Rev Phytopathol 8:231

Liu W, Chao Y, Liu S, Qian S (2003) Molecular cloning and characterization of a laccase gene from the basidiomycete *Fome lignosus* and expression in *Pichia pastoris*. Appl Microbiol Biotechnol 63:174–181

Lobos S, Larrain J, Salas L, Cullen D, Vicuna R (1994) Isozymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. J Gen Microbiol 140:2691–2698

Lobos S, Larrondo L, Salas L, Karahanian E, Vicuña R (1998) Cloning and molecular analysis of a cDNA and the Cs-mnp1 gene encoding a manganese peroxidase isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Gene 206:185–193

Lomascola E, Record I, Herpoe-Gimbert M, Delattre J, Robert L, Georis J, Dauvrin T, Sigoillot J-C, Asther M (2003) Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. J Appl Microbiol 94:618–624

Lomascolo A, Cayol JL, Roche M, Guo L, Robert JL, Record E, Lessage-Meessen L, Olliver B, Sigoillot JC, Asther M (2002) Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. Mycol Res 106:1193–1203

López M, Loera O, Guerrero-Olazarán M, Viader-Salvadó JM, Gallegos-López JA, Fernández FJ, Favela-Torres E, Viniegra-González G (2010) Cell growth and Trametes versicolor laccase production in transformed *Pichia pastoris* cultured by solid-state or submerged fermentations. J Chem Technol Biotechnol 85:435–440

Lundell T (1993) Ligninolytic system of the white rot fungus Phlebia radiata: Lignin model compound studies. Dissertation, University of Helsinki

Ma B, Mayfield MB, Gold MH (2003) Homologous expression system of Phanerochaete chrysosporium manganese peroxidase, using bialaphos resistance as a dominant selectable marker. Curr Genet 43:407–414

Machuca A, Ferraz A (2001) Hydrolytic and oxidative enzymes produced by white- and brown-rot fungi during *Eucalyptus grandis* decay in solid medium. Enzyme Microb Technol 29:386–391

Majcherczyk A, Johannes C, Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme Microb Technol 22:335–341

Mäkelä MR, Galkin S, Hatakka A, Lundell T (2002) Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. Enzyme Microb Technol 30:542–549

Mäkelä MR, Hilden K, Hatakka A, Lundell TK (2009) Oxalate decarboxylase of the white rot fungus Dichomitus squalens demonstrates a novel enzyme primary structure and non-induced expression on wood and in liquid cultures. Microbiology 155:2726–2738

Maltseva OV, Niku-Paavola M-L, Leontievsky AA, Myasoedova NM, Golovleva LA (1991) Ligninolytic enzymes of the white rot fungus *Panus tigrinus*. Biotechnol Appl Biochem 13:291–302

Manubens A, Avila M, Canessa P, Vicuna R (2003) Differential regulation of genes encoding manganese peroxidase (MnP) in the basidiomycete *Ceriporiopsis subvermispora*. Curr Genet 43:433–438

Martinez AT, Camarero S, Guillen F, Gutierrez A, Munoz C, Varela E, Martinez MJ, Barrasa JM, Ruel K, Pelayo JM (1994) Progress in biopulping of non-woody materials – chemical, enzymatic and ultrastructural aspects of wheat-straw delignification with ligninolytic fungi from the genus *Pleurotus*. FEMS Microbiol Rev 13:265–274

Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol 22:695–700

Mayfield MB, Kishi T, Alic M, Gold MH (1994) Homologous expression of recombinant manganese peroxidase in *Phanerochaete chrysosp*orium. Appl Environ Microbiol 60:4303–4309

McGuirl MA, Dooley DM (1999) Copper-containing oxidases. Curr Opin Chem Biol 3:138–144

Mendonça RT, Jara JF, González V, Elissetche JP, Freer J (2008) Evaluation of the white rot fungi Ganoderma australe and Ceriporiopsis subvermispora in biotechnological applications. J Ind Microbiol Biotechnol 35:1323–1330

Mercer DK, Iqbal M, Miller PGG, McCarthy AJ (1996) Screening actinomycetes for extracellular peroxidase activity. Appl Environ Microbiol 62:2186–2190

Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J Biol Chem 273:15412–15417

Mhlanga CYL (2001) Thermophilic lignin degrading enzymes from actinomycetes for biotechnological applications. MSc thesis, Rhodes University

Micales JA (1997) Localization and induction of oxalate decarboxylase in the brown-rot wood decay fungus *Postia pacenta*. Int Biodeter Biodegr 39:125–132

Moo-Young M, Chahal DS, Vlach D (1978) Single cell protein from various chemically pretreated wood substrates using *Chaetomium cellulolyticum*. Biotechnol Bioeng 21:1361–1371

Moo-Young M, Moreira AR, Daugulis AJ (1979) Economics of fermentation processes for SCP production from agricultural wastes. Can J Chem Eng 57:741–749

Moreira MT, Feijoo G, Lema JM (2000a) Manganese peroxidase production by *Bjerkandera* sp. BOS55.1. Regulation of enzymatic production. Bioprocess Eng 23:657–661

Moreira MT, Mielgo I, Feijoo G, Lema JM (2000b) Evaluation of different fungal strains in the decolorization of synthetic dyes. Biotechnol Lett 22: 1499–1503

Moreira MT, Torrado A, Feijoo G, Lema JM (2000c) Manganese peroxidase production by Bjerkandera sp. BOS55.2. Operation in stirred tank reactors. Bioprocess Eng 23:663–667

Moreira PR, Duez C, Dehareng D, Antunes A, Almeida-Vara E, Frère JM, Malcata FX, Duarte JC (2005) Molecular characterisation of a versatile peroxidase from a Bjerkandera strain. J Biotechnol 118:339–352

Moyson E, Verachtert H (1991) Growth of higher fungi on wheat straw and their impact on the digestibility of the substrate. Appl Microbiol Biotechnol 36:421–424

Muheim A, Waldner R, Leisola MSA, Fiechter A (1990) An extracellular aryl-alcohol oxidase from the white-rot fungus *Bjerkandera adusta*. Enzyme Microb Technol 12:204–209

Munoz C, Guillen F, Martinez AT, Martinez MJ (1997a) Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+} oxidation. Appl Environ Microbiol 63:2166–2174

Munoz C, Guillen F, Martinez AT, Martinez MJ (1997b) Induction and characterization of laccase in the lignin-olytic fungus *Pleurotus eryngii*. Curr Microbiol 34:1–5

Nagai M, Kawata M, Watanabe H, Ogawa M, Saito K, Takesawa T, Kanda K, Sato T (2003) Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. Microbiology 149:2455–2462

Nagai M, Sakamoto Y, Nakade K, Sato T (2007) Isolation and characterization of the gene encoding a manganese peroxidase from *Lentinula edodes*. Mycoscience 48:125–130

Nair LM (2006) Bioconversion of Prosopis juliflora (Vilayati babul) hydrolysate in to ethanol by *Pichia stipis* NCIM-3498. MPhil thesis, University of Delhi, New Delhi

Nerud F, Misurcova Z (1989) Production of ligninolytic peroxidases by the white-rot fungus *Coriolopsis occidentalis*. Biotechnol Lett 11:427–432

Nerud F, Zouchova Z, Misurcova Z (1991) Ligninolytic properties of different white-rot fungi. Biotechnol Lett 13:657–660

Nieves RA, Ehrman CI, Adney WS, Elander RT, Himmel ME (1998) Technical communication: survey and analysis of commercial cellulase preparation suitable for biomass conversion to ethanol. World J Microbiol Biotechnol 14:301–304

Nigam JN (1998) Single cell protein from pineapple cannery effluent. World J Microbiol Biotechnol 14:693–696

Niku-Paavola ML, Raaska L, Itavaara M (1990) Detection of white-rot fungi by a non-toxic stain. Mycol Res 94:27–31

Nilsson T, Daniel GF (1983) Tunnelling bacteria. Document, International Research Group/Wood Preservation, No. 1186. France

Nilsson T, Daniel GF, Kirk TK, Obst JR (1989) Chemistry and microscopy of wood decay by some higher ascomycetes. Holzforschung 43:11–18

Novotny C, Erbanova P, Cathaml T, Rothschild N, Dosoretz C, Sasek V (2000) *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. Appl Microbiol Biotechnol 54:850–853

Nuske J, Scheibner K, Dornberger U, Ullrich R, Hofrichter M (2002) Large scale production of manganese-peroxidase using agaric white-rot fungi. Enzyme Microb Technol 30:556–561

O'Callaghan J, O'Brien MM, McClean K, Dobson AD (2002) Optimization of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. J Ind Microbiol Biotechnol 29:55–59

O'Hara EB, Timberlake WE (1989) molecular characterization of the *Aspergillus nidulans* yA locus. Genetics 121:249–254

Okano K, Kitagawa M, Sasaki Y, Watanabe T (2005) Conversion of Japanese red cedar (*Cryptomeria japonica*) into a feed for ruminant by white-rot basidiomycetes. Anim Feed Sci Technol 120:235–243

Okano K, Iida Y, Samsuri M, Prasetya B, Usagava T, Watanabe T (2006) Comparison of in vitro digestibility and chemical composition among sugarcane bagasses treated by four white rot fungi. Anim Sci J 77:308–313

Okano K, Ohkoshi N, Nishiyama A, Usagawa T, Kitagawa M (2009) Improving the nutritive value of madake bamboo, *Phyllostachys bambusoides*, for ruminants by culturing with the white-rot fungus *Ceriporiopsis subvermispora*. Anim Feed Sci Technol 152:278–285

Orth AB, Royse DJ, Tien M (1993) Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl Environ Microbiol 59:4017–4023

Otjen L, Blanchette RA, Leatham GF (1988) Lignin distribution in wood delignified by white rot fungi-X-ray-microanalysis of decayed wood treated with bromine. Holzforschung 42:281–288

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G (1997) A novel white laccase from *Pleurotus ostreatus*. J Biol Chem 272:31301–31307

Palmieri G, Giardina P, Bianco C, Fontanella B, Sannia G (2000) Copper induction of laccase isoenzymes in the lignolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol 66:920–924

Papinutti VL, Diorio LA, Forchiassin F (2003) Production of laccase and manganese peroxidase by *Fomes sclerodermeus* grown on wheat bran. J Ind Microbiol Biotechnol 30:157–160

Pease EA, Tien M (1992) Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. J Bacteriol 174:3532–3540

Périé FH, Gold MH (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. Appl Environ Microbiol 57:2240–2245

Phillippi F (1893) Die Pilze Chiles, soweit dieselben als Nahrungsmittel gebraucht werden. Hedwigia 32:115–118

Pickard MA, Vandertol H, Roman R, Vazquez-Duhalt R (1999) High production of ligninolytic enzymes from white-rot fungi in cereal bran liquid medium. Can J Microbiol 45:627–631

Piscitelli A, Giardina P, Mazzoni C, Sannia G (2005) Recombinant expression of *Pleurotus ostreatus* laccases in Kluyveromyces *lactis* and *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 69:428–439

Pogni R, Baratto MC, Teutloff C, Giansanti S, Ruiz-Dueñas FJ, Choinowski T, Piontek K, Martínez AT, Lendzian F, Basosi R (2006) A tryptophan neutral radical in the oxidized state of versatile peroxidase from *Pleurotus eryngii*: a combined multifrequency

EPR and density functional theory study. J Biol Chem 281:9517–9526
Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57: 20–33
Pointing SB, Jones EBG, Vrijmoed LLP (2000) Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia 92:139–144
Raeder U, Thompson W, Broda P (1989) RFLP-based genetic map of *Phanerochaete chrysosporium* ME446: lignin peroxidase genes occur in clusters. Mol Microbiol 7:911–918
Rajakumar S, Gaskell J, Cullen D, Lobos S, Karahanian E, Vicuna R (1996) *liP*-like genes in *Phanerochaete sordida* and *Ceriporiopsis subvermispora*, white rot fungi with no detectable lignin peroxidase activity. Appl Environ Microbiol 62:2660–2663
Ralph JP, Graham LA, Catcheside DEA (1996) Extracellular oxidases and the transformation of solubilised low-rank coal by wood-rot fungi. Appl Microbiol Biotechnol 46:226–232
Ramachandra M, Crawford DL, Hertel G (1988) Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete Streptomyces viridosporus. Appl Environ Microbiol 54:3057–3063
Rayner ADM, Boddy L (1988) Fungal decomposition of wood. Wiley, London
Record E, Punt PJ, Chamkha M, Labat M, van den Hondel CAMJJ, Asther M (2002) Expression of the Pycnoporus cinnabarinus laccase gene in Aspergillus niger and characterization of the recombinant enzyme. Eur J Biochem 269:602–609
Reddy GV, Babu PR, Komaraih P, Roy KRRM, Kothari IL (2003) Utilization of banana waste for the production of ligninolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor-caju*). Process Biochem 38:1457–1462
Reinhammar B (1984) Laccase. In: Lontie R (ed) Copper proteins and copper enzymes. CRC Press, Boca Raton, pp 1–35
Ricotta A, Unz RF, Bollag JM (1996) Role of laccase in the degradation of pentachlorophenol. Bull Environ Contam Toxicol 57:560–567
Riva S (2006) Laccases: blue enzymes for green chemistry. Trends Biotechnol 24:219–226
Robene-Soustrade I, Lung-Escarmant B, Bono JJ, Taris B (1992) Identification and partial characterization of an extracellular manganese-dependent peroxidase in *Armillaria ostoyae* and *Armillaria mellea*. Eur J Forest Pathol 22:227–236
Robertson SA, Mason SL, Hack E, Abbott GD (2008) A comparison of lignin oxidation, enzymatic activity and fungal growth during white-rot decay of wheat straw. Org Geochem 39:945–951
Robinson T, Chandran B, Nigam P (2001) Studies on the production of enzymes by white-rot fungi for the decolorisation of textile dyes. Enzyme Microb Technol 29:575–579
Rodríguez E, Ruiz-Dueñas FJ, Kooistra R, Ram A, Martínez AT, Martínez MJ (2008) Isolation of two laccase genes from the white-rot fungus Pleurotus eryngii and heterologous expression of the pel3 encoded protein. J Biotechnol 134:9–19
Romero E, Speranza M, García-Guinea J, Martínez AT, Martínez MJ (2007) An anamorph of the white-rot fungus Bjerkandera adusta capable of colonizing and degrading compact disc components. FEMS Microbiol Lett 275:122–129
Rothschild N, Hadar Y, Dosoretz CG (1997) Lignin peroxidase isozymes from *Phanerochaete chrysosporium* can be enzymatically dephosphorylated. Appl Environ Microbiol 63:857–861
Ruiz-Dueñas FJ, Martinez MJ, Martinez AT (1999) Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*. Mol Microbiol 31:223–235
Ruiz-Duenas FJ, Camarero S, Perez-Boada M, Martınez MJ, Martınez AT (2001) A new versatile peroxidase from *Pleurotus*. Biochem Soc Trans 29:116–122
Ruttiman GB, Vicuna R, Sapag C, Seenlenfreund D (1998) Biochemical and genetic studies of bacteria metabolizing lignin-related compounds. Arch Biol Med Exp 21:247–255
Ruttimann C, Schwember E, Salas L, Cullen D, Vicuna R (1992) Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnol Appl Biochem 16:64–76
Sakamoto Y, Nakade K, Nagai M, Uchimiya H, Sato T (2009) Cloning of Lentinula edodes lemnp2, a manganese peroxidase that is secreted abundantly in sawdust medium. Mycoscience 50:116–122
Saloheimo M, Barajas V, Niku-Paavola M-L, Knowles JKC (1989) A lignin peroxidase-encoding cDNA from the white rot fungus *Phlebia radiata*: characterization and expression in *Trichoderma reesei*. Gene 85:343–351
Saloheimo M, Leena M, Paavola N (1991) Heterologous production of a lignolytic enzyme: expression of the *Phlebia radiate* laccase gene in *Trichoderma reesei*. Nat Biotechnol 9:987–990
Sánchez C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185–194
Saparrat MCN, Guillén F, Arambarri AM, Martínez AT, Martínez MJ (2002) Induction, isolation and characterization of two laccases from the white rot basidiomycete Coriolopsis rigida. Appl Environ Microbiol 68:1534–1540
Schmidt O, Nagashima Y, Liese W, Schmitt U (1987) Bacterial wood degradation studies under laboratory conditions in lakes. Holzforschung 41:137–140
Schoemaker HE, Leisola MSA (1990) Degradation of lignin by *Phanerochaete chrysosporium*. J Biotechnol 13:101–109
Sermanni GG, D'Annibale A, Di Lena G, Vitale NS, Di Mattia E, Minelli V (1994) The production of exoenzymes by *Lentinus edodes* and *Pleurotus ostreatus* and their use for upgrading corn straw. Bioresour Technol 48:173–178

Shah MP, Reddy GV, Banerjee R, Babu PR, Kothari IL (2005) Microbial degradation of banana waste under solid state bioprocessing using two lignocellulolytic fungi (*Phylosticta* spp. MPS-001 and *Aspergillus* spp. MPS-002). Process Biochem 40:445–451

Sharma RK, Arora DS (2010a) Production of lignocellulolytic enzymes and enhancement of in vitro digestibility during solid state fermentation of wheat straw by *Phlebia floridensis*. Bioresour Technol 101:9248–9253

Sharma RK, Arora DS (2010b) Changes in biochemical constituents of paddy straw during degradation by white rot fungi and its impact on in vitro digestibility. J Appl Microbiol 109:679–686

Sharma KK, Kuhad RC (2010) Genetic transformation of lignin degrading fungi facilitated by *Agrobacterium tumefaciens*. BMC Biotechnol 10:67–75

Sharma KK, Kapoor M, Kuhad RC (2005) In vivo enzymatic digestion, in vitro xylanase digestion, metabolic analogues, surfactants and polyethylene glycol ameliorate laccase production from *Ganoderma* sp. kk-02. Lett Appl Microbiol 41:24–31

Sharma KK, Gupta S, Kuhad RC (2006) *Agrobacterium*-mediated delivery of marker genes to *Phanerochaete chrysosporium* mycelial pellets: a model transformation system for white-rot fungi. Biotechnol Appl Biochem 43:181–186

Shary S, Ralph SA, Hammel KE (2007) New insights into the ligninolytic capability of a wood decay ascomycete. Appl Environ Microbiol 20:6691–6694

Shrivastava B, Thakur S, Khasa YP, Gupte A, Puniya AK, Kuhad RC (2011) White-rot fungal conversion of wheat straw to energy rich cattle feed. Biodegradation 22:823–831

Sigoillot C, Camarero S, Vidal T, Record E, Asther M, Pérez-Boada M, Martínez MJ, Sigoillot J-C, Asther M, Colom JF (2005) Comparison of different fungal enzymes for bleaching high-quality paper pulps. J Biotechnol 115:333–343

Singh A, Abidi AB, Darmwal NS, Agrawal AK (1991) Influence of nutritional factors on cellulase production from natural lignocellulosic residues by *Aspergillus niger*. Agric Biol Res 7:19–27

Singh A, Bajar S, Bishnoi NR, Singh N (2010) Laccase production by *Aspergillus heteromorphus* using distillery spent wash and lignocellulosic biomass. J Hazard Mater 15:79–82

Sirohi SK, Rai SN (1999) Synergistic effect of urea and lime treatment of wheat straw on chemical composition, in sacco and in vitro digestibility. Asian Aust J Anim Sci 12:1049–1053

Sjoblad RD, Bollag JM (1981) Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In: Paul EA, Ladd JN (eds) Soil biochemistry. Marcel Dekker, New York, pp 113–152

Steffen KT (2003) Degradation of recalcitrant biopolymers and polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Dissertationes Biocentri Viikki Universitatis Helsingiensis, 23/2003. PhD thesis, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, p 68

Steffen KT, Hofrichter M, Hatakka A (2002a) Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. Enzyme Microb Technol 30:550–555

Steffen KT, Hatakka A, Hofrichter M (2002b) Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. Appl Environ Microbiol 68:3442–3448

Stewart CS, Bryant MP (1988) The rumen bacteria. In: Hobson PN (ed) The rumen microbial ecosystem. Elsevier, New York, pp 21–76

Stewart P, Kersten P, Wymelenberg AV, Gaskell J, Cullen D (1992) Lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation and identification of a second dimorphic chromosome. J Bacteriol 174: 5036–5042

Stewart P, Whitwam RE, Kersten PJ, Cullen D, Tien M (1996) Efficient expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oryzae*. Appl Microbiol Biotechnol 62:860–864

Straatsma G, Samson RA, Olijnsma TW, Op den Camp HJM, Gerrits JPG, van Griensven LJLD (1994) Ecology of thermophilic fungi in mushroom compost, with emphasis on *Scytalidium thermophilum* and growth stimulation of *Agaricus bisporus* mycelium. Appl Environ Microbiol 60:454–458

Sugiura T, Yamagishi K, Kimura T, Nishida T, Kawagishi H, Hirai H (2009) Cloning and homologous expression of novel lignin peroxidase genes in the white-rot fungus *Phanerochaete sordida* YK-624. Biosci Biotechnol Biochem 73:1793–1798

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11

Sundaramoorthy M, Kishi K, Gold MH, Poulos TL (1994) The crystal structure of manganese peroxidase from Phanerochaete chrysosporium at 2.06-A resolution. J Biol Chem 269:32759–32767

Sundstol F, Owen E (1984) Straw and other fibrous by-products as feed. Elsevier, Amsterdam, 604

Sutherland JB, Blanchette RA, Crawford DL, Pometto AL (1979) Breakdown of Douglas-fir phloem by a lignocellulose-degrading *Streptomyces*. Curr Microbiol 2:123–126

Suzuki Y, Okano K, Kato S (1995) Characteristics of white-rotted woody materials obtained from shiitake mushroom (*Lentinus edodes*) and nameko mushroom (*Pholiota nameko*) cultivation with in vitro rumen fermentation. Anim Feed Sci Technol 24:130–137

Suzuki T, Endo K, Ito M, Tsujibo H, Miyamoto K, Inamori Y (2003) A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. Biosci Biotechnol Biochem 67:2167–2175

Szklarz GD, Antibus RK, Sinsabaugh RL, Linkins AE (1989) Production of Phenol oxidases and Peroxidases by wood-rotting fungi. Mycologia 8:234–240

Tagger S, Perissol C, Gil G, Vogt G, Le Petit J (1998) Phenoloxidases of the white-rot fungus *Marasmius quercophilus* isolated from an evergreen oak litter (*Quercus ilex* L.). Enzyme Microb Technol 23: 372–379

Tamaru H, Inoue H (1989) Isolation and characterization of a laccase-depressed mutant of Neurospora crassa. J Bacteriol 171:6288–6293

Tekere M, Zvvauya R, Read JS (2001) Ligninolytic enzyme production in selected sub-tropical white rot fungi under different culture conditions. J Basic Microbiol 41:115–129

Temp U, Zierold U, Claudia E (1999) Cloning and characterization of a second lactase gene from the lignin-degrading basidiomycete *Pycnoporus cinnabarinus*. Gene 236:169–177

Ten Have R, Teunissen PJM (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. Chem Rev 101:3397–3413

Teunissen PJM, Field JA (1998) 2-Chloro-14-dimethoxybenzene as a novel catalytic cofactor for oxidation of anisyl alcohol by lignin peroxidase. Appl Environ Microbiol 64:830

Thomke S, Rundgren M, Eriksson S (1980) Nutritional evaluation of the white rot fungus – *Sporotrichum pulverulentum* – as a feedstuff to rats, pigs and sheep. Biotechnol Bioeng 22:2285–2303

Tien M, Kirk TK (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science 221:661–663

Tien M, Tu C-PD (1987) Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*. Nature 326:520–523

Tien M, Kirk TK, Bull C, Fee JA (1986) Steady-state and transient-state kinetic studies on the oxidation of 3, 4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerocheate chrysosporium* Burds. J Biol Chem 261:1687–1693

Touchburn SP, Chavex ER, Moo-Young M (1986) *Chaetomium cellulolyticum* microbial biomass protein evaluation with rats, chicks and piglets. In: Moo-Young M, Gregory KF (eds) Microbial biomass proteins. Elsevier Applied Science, London, pp 175–185

Tripathi JP, Yadav JS (1992) Optimisation of solid substrate fermentation of wheat straw into animal feed by *Pleurotus ostreatus* – a pilot effort. Anim Feed Sci Technol 37:59–72

Tsukihara T, Honda Y, Sakai R, Watanabe T, Watanabe T (2006) Exclusive overproduction of recombinant versatile peroxidase MnP2 by genetically modified white rot fungus, *Pleurotus ostreatus*. J Biotechnol 126:431–439

Tuncer M, Ball AS (2002) Degradation of lignocellulose by extracellular enzymes produced by *Thermomonospora fusca* BD25. Appl Microbiol Biotechnol 58:608–611

Urzúa U, Larrondo LF, Lobos S, Larraín J, Vicuña R (1995) Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. FEBS Lett 371:132–136

Valaskova V, Baldrian P (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* – production of extracellular enzymes and characterization of the major cellulases. Microbiology 152:123613–123622

Valdez ODM, Flores EOG, García JAM et al (2008) Use of *Pleurotus pulmonarius* to change the nutritional quality of wheat straw. I. Effect on chemical composition. Interciencia 33:435–438

Varela E, Martinez AT, Martinez MJ (2000) Southern blot screening for lignin peroxidase and aryl-alcohol oxidase genes in 30 fungal species. J Biotechnol 83:245–251

Vares T (1996) Ligninolytic enzymes and lignin-degrading activity of taxonomically different white-rot fungi. PhD thesis, vol 44, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland, p 67

Vares T, Lundell TK, Hattaka AI (1992) Novel heme-containing enzyme possibly involved in lignin degradation by the white-rot fungus *Junghuhnia separabilima*. FEMS Microbiol Lett 99:53–58

Vares T, Lundell TK, Hattaka AI (1993) Production of multiple lignin peroxidases by the white-rot fungus *Phlebia ochraceofulva*. Enzyme Microb Technol 15:664–669

Vares T, Niemenmaa O, Hatakka A (1994) Secretion of ligninolytic enzymes and mineralization of 14 C-labelled synthetic lignin by three Phlebia tremellosa strains. Appl Environ Microbiol 60:569–575

Vasdev K, Kuhad RC (1994) Induction of laccase production in *C. bulleri* under shaking and static culture conditions. Folia Microbiol 39:326–330

Vasdev K, Dhawan S, Kapoor KR, Kuhad RC (2005) Biochemical characterization and molecular evidence of a laccase from the birds nest fungus C*yathus bulleri*. Fungal Genet Biol 42:684–693

Vikineswary S, Abdullah N, Renuvathani M, Sekaran M, Pandey A, Jones EBG (2006) Productivity of laccase in solid substrate fermentation of selected agro-residues by *Pycnoporus sanguineus*. Bioresour Technol 97:171–177

Villas-Boas SG, Esposito E, Mitchell DA (2002) Microbial conversion of lignocellulosic residues for production of animal feeds. Anim Feed Sci Technol 98:1–12

Villas-Boas SG, Esposito E, De Mendonca MM (2003) Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus*. Word J Microbiol Biotechnol 19:461–467

Volc J, Kubatova E, Daniel G, Sedmera P, Haltrich D (2001) Screening of basidiomycete fungi for the quinone-dependent sugar C-2/C-3 oxidoreductase, pyranose dehydrogenase, and properties of the enzyme from *Macrolepiota rhacodes*. Arch Microbiol 176:178–186

Von Hunolstein C, Valenti CP, Visca P, Antonini G, Nicolini L, Orsi N (1986) Production of laccases A and B by a mutant strain of Trametes versicolor. J Gen Appl Microbiol 32:185–191

Wahleithner JA, Xu F, Brown KM, Brown SH, Golightly EJ, Halkier T, Kauppinen S, Pederson A, Schneider P (1996) The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. Curr Genet 29:395–403

Waldner R, Leisola MSA, Fiechter A (1988) Comparison of ligninolytic activities of selected white-rot fungi. Appl Microbiol Biotechnol 29:400–407

Walker HG (1984) Physical treatment. In: Straw and other fibrous byproducts as feed. Elsevier, Amsterdam, pp 79–101

Wariishi H, Valli K, Renganathan V, Gold MH (1989) Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J Biol Chem 264:14185–14191

Watanabe Y, Shinzato N, Fukatsu T (2003) Isolation of actinomycetes from termites' guts. Biosci Biotechnol Biochem 67:1797–1801

Wong DWS (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 157:174–209

Wood PM (1994) Pathways for production of Fenton's reagent by wood-rotting fungi. FEMS Microbiol Rev 13:313–320

Yadav JS, Tripathi JP (1991) Optimization of cultivation and nutrition conditions and substrate pretreatment for solid-substrate fermentation of wheat straw by *Coriolus versicolor*. Folia Microbiol 36:249–301

Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalboge H (1996) Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol 62:834–841

Yeo S, Park N, Song H, Choi HT (2007) Generation of a transformant showing higher manganese peroxidase (Mnp) activity by overexpression of Mnp gene in *Trametes versicolor*. J Microbiol 45:213–218

Zadrazil F (1985) Screening of fungi for lignin decomposition and conversion of straw into feed. Angew Bot 59:433–452

Zadrazil F, Puniya AK (1995) Studies on effect of particle size on solid state fermentation of sugar cane bagasse into animal feed using white-rot fungi. Bioresour Technol 54:85–87

Zadrazil F, Kamra DN, Isikhuemhen OS, Schuchardt F, Flachowsky G (1996) Bioconversion of lignocellulose into ruminant feed with white rot fungi. J Appl Anim Res 10:105–124

Zeng GM, Yu M, Chen YN, Huang DL, Zhang JC, Huang HL et al (2010) Effects of inoculation with *Phanerochaete chrysosporium* at various time points on enzyme activities during agricultural waste composting. Bioresour Technol 101:222–227

Zimmermann W (1990) Degradation of lignin by bacteria. J Biotechnol 13:119–130

Solid-State Bioconversion and Animal Feed Production: Present Status and Future Prospects

2

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Abstract

An overview of solid-state bioprocesses and its relevance to India is presented. The latest developments, existing problems, and future areas of research arising in methods of analysis, process optimization and scale-up, new reactor designs, instrumentation and control of solid-state bioprocesses, and systems analysis for screening and strain improvement have been discussed in detail. This chapter brings forth the need to integrate the biological and engineering sciences to catalyze the progress in this field to the next level where quantitative analysis, accuracy, and standardization will be achieved.

Keywords

Solid-state bioconversion • Animal feed • Lignocellulosic residues • Solid-state reactors • Optimization • Scale-up

Introduction

Lignocellulosic residue is a vast renewable energy resource abundantly available. About 2×10^{11} metric tons of carbon and 3×10^{13} J of energy (ten times the total energy presently consumed in the world) are fixed annually by photosynthesis in green plants. Agricultural residues comprise a substantial component of this in the form of cellulose, hemicellulose, and lignin. Lignocellulosic residues contain about 70% carbohydrates that can be used effectively and economically in animal feed preparations, provided some measures are taken to improve its utilization. Lignocellulose in wood may be transformed into paper products with the help of solid-state bioconversion (SSB), biopulping, and biobleaching processes. Agricultural residues may be converted into animal feed enriched with microbial biomass, enzymes, and biopromoters and made more digestible by SSB (Villas-Bôas et al. 2002). Lignocellulosic waste may be composted for the manufacture of biofertilizer, biopesticide, and biopromoter products. Postharvest residue may be decomposed on site by filamentous fungi and recycled into the soil

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with improved biofertilizer and bioprotective properties. In this chapter, the state of the art of lignocellulose bioconversion has been presented—the microbes used in the process, the fermentation technology with its engineering aspects, the main products of the bioconversion, and future trends in practical applications.

Most of the carbohydrates fractions in lignocellulosic residues are not available for utilization because of the presence of the recalcitrant lignin molecule. Physical or mechanical treatments, such as milling, grinding, chopping, and steaming, have long been used to improve the feed value (digestibility) of lignocellulosic residues (da Costa Souza et al. 2009; Bhatnagar et al. 2008). However, the small effect in improving digestibility by physical treatment is mainly due to the increase in surface area. Chemical methods have also been used, but with limited success, to improve the digestibility of lignocellulosic residues (Sahoo et al. 2002; Kumar and Gomes 2008). Alkaline agents can chemically break the ester bonds between lignin and hemicellulose and cellulose. Alkali makes the structural fibers swell thus enabling rumen microbes to attack the structural carbohydrates more easily, thereby improving digestibility. However, chemical methods require large volumes of water for removal of chemicals after treatment and add to the already existing problems of chemical pollutants and treatment cost. In addition, it increases the risk of exposing cattle to the danger of latent chemicals in the feed. Among the methods employed, the most promising results have been obtained from biological methods of degrading lignin. The use of intact microorganisms or enzymes produced by them, for the conversion of lignocellulosic residues into animal feed, has been an active area of research (Howard et al. 2003; Shrivastava et al. 2011; Goff et al. 2012).

Solid substrate bioconversion (SSB) is an important process applicable to most kinds of plant biomass. Lignocellulose, the primary constituent of plant biomass, is a feedstock that has been exploited for the production of biofuels, enzymes, and other biochemical products. Crop residues (straw, corn by-products, bagasse, etc.) are particularly suitable for this purpose, since they are available in large quantities in processing facilities. SSB may be defined as a process where microorganisms or enzymes act upon insoluble solid materials derived from natural resources, in the absence of free flowing liquid. Due to low-moisture content, bioconversion can only be effectively carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used (Singhania et al. 2009). As a major research topic, however, SSB process has not received much attention in the past, and the major efforts were focused on submerged fermentation (SmF). A closer evaluation of these two processes in recent years in several research centers throughout the world has revealed that for certain processes, there are enormous economical and practical advantages of SSB over SmF (Gamarra et al. 2010). These include non-aseptic conditions, use of economical raw materials as substrates, use of a wide variety of matrices (varied composition, size, mechanical resistance, porosity, and water holding capacity), low capital cost, low energy expenditure, lesser solvent for downstream processing, lesser water usage and lower wastewater output, and higher product concentration.

Solid-State Bioconversion for Animal Feed Production

The utilization of solid-state bioprocesses for converting lignocellulosic materials to animal feed has been practiced for more than five decades. However, the literature reveals that development of processes in this field has not integrated the microbiological and engineering sciences to yield what may be called *animal feed production technology*. A recent review restates this problem (Ajila et al. 2012). Other reviews also acknowledge the existence of problem in the development of a viable technology for animal feed production and examine relevant issues (Chiesa and Gnansounou 2011). There is a general agreement that the first period of development of solid-state bioprocesses took place between 1970 and 1975 and the second period of development took place between 1990 and 1999.

There are many important issues related to nutrition, palatability, large-scale production, and process economics that have never been adequately addressed in the literature. In particular, these issues are relevant, important, and of far-reaching consequence in the context of Indian scenario.

Relevance to India

There has been a gradual depletion of grazing land for cattle in India because of the tremendous population pressure on agricultural land. Although the impact of this trend has not yet been felt by farmers holding cattle for irrigation and milk production, a simple extrapolation shows that there will be insufficient green fodder in the near future. This in turn will affect various industries dependent on cattle and cattle products. In particular, it is a matter of concern that the dairy industry may be adversely affected. Although the dairy industry has been generating substantial revenue, to maintain their present turnover, it would be necessary to ensure alternative sources and alternative methods of fodder production. Insufficient fodder is already a problem in arid regions in India, especially in peak summer months. Therefore, developments in the technology for large-scale production of animal feed are needed.

Current Status and Future Prospects

Methods of Analysis

The variables identified and measured to characterize solid-state bioprocesses are lignin, cellulose, and hemicellulose degradation; oxygen uptake rate; and the temperature and humidity of the inlet and exit gases from the reactor. The quantitative methods of analysis for lignin, cellulose, and hemicellulose degradation are the methods that were developed in the 1970s (Updegraff 1969; Morrison 1972). These methods are cumbersome to carry out and are prone to large experimental errors. If these problems are addressed, and faster and accurate methods are developed, it will have a significant impact on scale-up and engineering improvements. Reports on the development of new methods of lignin and cellulose analysis show that researchers have recognized the magnitude of this problem (Agblevor et al. 1994; Dupont and Mortha 2004; Ohra-aho et al. 2005; Scarlata and Hyman 2010; Goff et al. 2012).

The measurement of oxygen uptake rate is well established and may be carried as routinely as it is done in submerged cultivation. Analysis of inlet and exit gases using paramagnetic oxygen sensors and infrared carbon dioxide sensors can give accurate measurements of the oxygen and carbon dioxide concentrations and their rates of utilization and evolution, respectively. An important precaution especially applicable in SSB is to ensure that all the moisture is removed from the sample gas before it reaches the probes. Humidity measurements of the inlet and exit gas streams may also be made using humidity probes. Since these probes are prone to giving saturated readings if condensation occurs on the active element, correct direction of the airflow stream is necessary to ensure that trouble-free online monitoring of humidity.

Process Optimization and Scale-Up

Solid-state bioprocesses are three-phase processes and are difficult to optimize experimentally. Theoretical methods of optimization require first a detailed and accurate model of the process and extensive mathematical analysis to determine the optimum in terms of maximizing or minimizing a given objective function. Many practical problems can only be described qualitatively; hence, only partial solutions from the perspective of the individual researcher appear in the literature (Basu et al. 2002; Raghavrao et al. 2003; Kumar and Gomes 2008). A complete optimization would incorporate both experimental and mathematical treatments in reasonable detail and the application of statistical methods for the evaluation of performance of the optimization exercise. The optimization exercise itself will depend

on the type of reactor and the scale of operation. Therefore, the future of this area of research in terms of underutilized information and its potential application in both existing and new processes is far reaching. What is required at this point is a proposition of an acceptable exhaustive optimization methodology and proof of its success in a process environment.

One such proposition outlined below considers the experimental plan in which the seed culture stage is also included and a theoretical plan that includes modeling and mathematical determination of the optimum. The reactor performance is analyzed statistically:

1. Design experiments based on statistical design methods for carrying out SSB in which the effect of a critical variable, for example, the biomass of inoculum, is accounted for.
2. Write detailed models and identify parameters of the model (Mitchell et al. 2000). Often simple models may give the required information being sought, and in such cases, the simpler model should be accepted. In addition, one may consider the development of the model based on variables, such as lignin degradations (or content) that can be directly measured and offer better process understanding.
3. Describe an objective function for the process considering market forces. Compute either numerically or analytically the optimum conditions.
4. Perform experiments and verify the predictions from experimental and mathematical optimization.
5. Analyze reactor or process performance based on productivity and profit considerations.

Scale-up follows optimization and pertains to the exercise that converts production of small volumes to large volumes. Normally, it is based on one of the variables to which the performance of the reactor exhibits high sensitivity. A detailed analysis has been presented by Mitchell et al. (2000). In their analysis, temperature plays an important role. Clearly, other variables, such as lignin degradation, that also have an impact on performance cannot be included until issues related to accurate measurement of lignin are resolved. Bed height and particle size are physical variables that strongly influence performance because of the effect these variables exert on mass and heat transfer (Mitchell et al. 2000; Valera et al. 2005). Above all, scale-up and design must address practical issues of enabling conductive and convective heat transfer, adequate mass transfer, and material handling (loading and unloading) convenience (Suryanarayan and Mazumdar 2001; Valera et al. 2005). Often, these cannot be achieved without sacrificing an advantage. Further, what is desirable is the development of a procedure using dimensionless variables and is a parallel of that which exists for submerged cultivation bioprocesses (Hardin et al. 2000).

Engineering Problems Associated with Solid-State Bioconversion

The reasons why SSB has not yet found a broad use in India are related to the engineering issues, mainly the low amenability of the processes to standardization and the limited reproducibility of the results. Scale-up represents a particular bottleneck because several different parameters (e.g., temperature, humidity, substrate concentration), which can arise during the course of the process, can have an adverse effect not only in static solid bed processes but also in processes involving reactor content mixing, such as those performed in rotating drums. Interacting relationships among environmental factors such as oxygen content, moisture level, and temperature contribute to the difficult regulation of these parameters. The microbial growth under aerobic conditions in the bioreactor results in a considerable production of heat that causes rapid increase in temperature. This effect, which results in hotspots, is undesirable in bioconversion because the normal temperature of operation is below 40°C, and there exists a danger of products being heat denatured. In the absence of a free aqueous phase, the produced heat is difficult to remove, for example, via the bioreactor double walls. Instead, the cooling of the process takes place through evaporation. This requires very high aeration rates that increase with increasing metabolic activity. Since the reactor contents in a SSB

processes are poor heat conductors, high aeration rates needed for heat removal usually overcompensate for the heat produced within the reactor. This results in loss of humidity, and the water lost must be replenished by spray or mist devices. In a large-scale reactor, water replenishment can cause local condensation that adversely affects reactor performance. In semi-sterile processes, increased water activity can in turn facilitate the growth of bacterial contaminants, whereas in sterile fermentations, it may create local anoxic zones detrimental to the proliferation of microorganisms. Substrate mixing may help, but it is not recommended because many microorganisms respond very sensitively to the shear stress caused by it. Another factor that is difficult to account for is the production of metabolic water by aerobic microorganisms, which can cause problems especially in the formation of conidiospores (Rahardjo et al. 2006).

Reactor Designs

The design of an efficient industrial-scale reactor for SSB is of significance because it produces less effluent than SmF. However, it shows considerable drawbacks such as heat and mass transfer resistance, steep gaseous concentration, and thermal gradients that develop within the medium bed, which may adversely affect solid-state fermenter performances. Agitation and rotation in SSF were often carried out to improve mass and heat transfers, but the shearing force caused by agitation and rotation has adverse effects on medium porosity and disrupts fungal mycelia.

There are four types of reactors used in SSB processes, and each of these designs possesses features that favor certain types of SSB process conditions. The bioreactors commonly used, which can be distinguished by the type of aeration or the mixing system employed, include the following:

Tray: It consists of an ensemble of flat trays. The substrate is spread onto each tray forming a thin layer, only a few centimeters deep. The reactor is kept in a chamber at constant temperature through which humidified air is circulated. The main disadvantage of this configuration is that numerous trays and large floor area are required, making it an unattractive design for large-scale production.

Packed bed: It is usually composed of a column made of plastic, glass, or steel with the solid substrate retained on a perforated base. Through the bed of substrate, humidified air is continuously forced (Durand et al. 1993; Raimbault 1998; Rodríguez Couto et al. 2000). It may be fitted with a jacket for circulation of water to control the temperature during fermentation. This is the configuration usually employed in commercial koji production. The main drawbacks associated with this configuration are the following: difficulties in obtaining the product, nonuniform growth, poor heat removal, and scale-up problems.

Horizontal drum: This design allows adequate aeration and mixing of the substrate, whilst limiting the damage to the inoculum or product. Mixing is performed by rotating the entire vessel or by various agitation devices such as paddles and baffles (Domínguez et al. 2001; Nagel et al. 2001a, b; Prado et al. 2004; Stuart et al. 1999). Its main disadvantage is that the drum is filled to only 30% capacity; otherwise, mixing is inefficient.

Fluidized bed: In order to avoid the adhesion and aggregation of substrate particles, this design provides continuous agitation with forced air. Although the mass heat transfer, aeration, and mixing of the substrate are increased, damage to inoculum through a sheer forces and lower fluidity of material under high humidity conditions may affect the final product yield.

The production of animal feed from lignocellulosic residues in various types of bioreactors has been studied at the laboratory scale and pilot scale (Dasthban et al. 2009; Kumar and Gomes 2008; Bhatnagar et al. 2008). Designing of large-scale bioreactors for solid-state bioconversion (SSB) is different from the design of submerged reactors due to difference in physical characteristics of the medium. It is important to carry out the bioconversion under conditions such that

lignin is degraded as much as possible with minimum utilization of cellulose. In this way, the energy content of the residue is preserved, and the cellulose is exposed for easy digestion in the rumen of the animal. The heat transfer with the solid substrate is one of the major problems of the SSB due to poor conductivity of the lignocellulosic residues (Ashley et al. 1999; Raghavrao et al. 2003; Singhania et al. 2009). The metabolically generated heat during growth creates spatial temperature gradients. Mass transfer is another problem of the SSB process. During scale-up of bioreactors for animal feed production by SSB process, heat and mass transfer problems are major considerations.

The history of design of reactors for bioconversion of lignocellulosic residues to various value products is more than 50 years old. The current status of industrial implementation is constrained to a maximum processing of only a few tons of raw materials. Traditionally, rotary drum reactors have been used for solid-state bioconversion. However, problems of material handling, nonuniform mixing, and low overall yield in these reactors are well known. This had initiated the research for new designs more than two decades ago. Several new designs have also been implemented by the industry, among which the Plafractor designed by Biocon Ltd., India, has been particularly successful (Suryanaryan and Mazumdar 2001). Bhatnagar et al. (2008) developed operating conditions for a 200 L staged vertical reactor for bioconversion of wheat straw by *Phanerochaete chrysosporium*. Kumar and Gomes (2008) and Gomes et al. (2006) developed a vertical reactor, and its performance evaluation was reported for animal feed production. No single reactor design can solve all the problems faced in solid-state bioconversion processes and can provide solutions only to particular problems.

Among the many engineering considerations, the ones that strongly influence the design and performance analysis of reactors are (1) provisions for material handling, (2) accuracy of temperature control, (3) accuracy of humidity control, (4) efficiency of in situ sterilization, (5) efficiency of air circulation and nutrient supply, and (6) ease of reactor cleaning and maintenance. An example of chronology of reactor design from laboratory scale to pilot scale for the bioconversion of lignocellulosic residues to animal feed can take years of development (see Fig. 2.1).

Fig. 2.1 1,200 L packed bed bioreactor. The reactor is a stand-alone unit and operates in all weather conditions

Instrumentation and Control of Solid-State Bioprocesses

The primary difficulty that faces an engineer implementing instrumentation and control for SSB is the heterogeneity of the solid matrix and the poor conductivity of lignocellulosic materials. Along with these difficulties, the three-phase SSBs cannot be provided with instrumentation to measure directly variables such as lignin and cellulose content. The only variables that seem to be amenable to instrumentation are humidity and temperature. Consequently, control of SSBs will continue to be a challenge to the control engineer.

Temperature and humidity, along with mixing (agitation speed) and airflow rate, can be

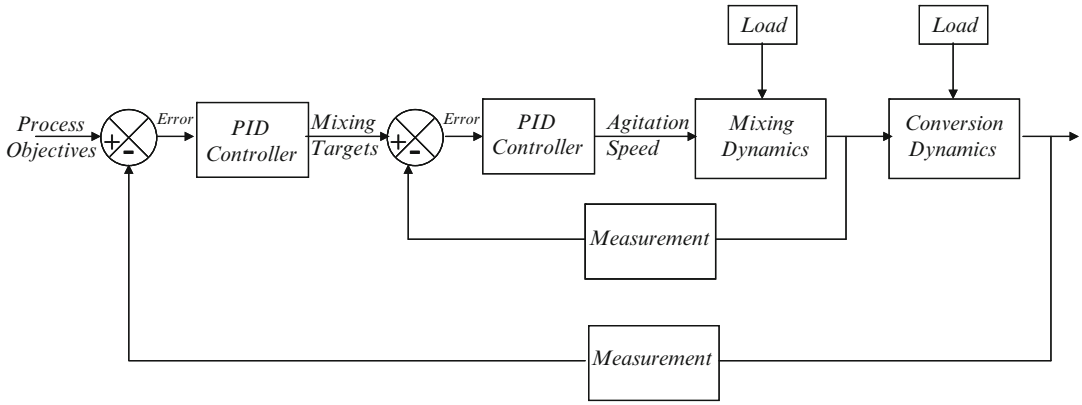

Fig. 2.2 A general cascade control loop applied to solid-state bioprocesses

independently controlled by feedback PID (proportional-integral-derivative) controllers, and at best, cascade controllers may be implemented (Fig. 2.2). Although the classical PID controllers can be used for controlling simple loops, it is not possible to implement goal-oriented time-varying trajectories in solid-state bioprocesses. Advanced control strategies are required. Considering the complexity of modeling three-phase systems in which partial derivatives appear as a regular feature, the application of nonlinear control strategies may not be straightforward, and considerable theoretical developments will be required. However, it will definitely be easier to develop Artificial Neural Networks for prediction and control of solid-state bioprocesses (Nayak and Gomes 2006). In particular, the development of continuous solid-state bioprocesses will mean that this area of research can no longer be ignored.

Systems Analysis for Screening and Strain Improvement

The environment contains a myriad of microorganisms, few of which may be satisfactory with respect to a desired purpose. The diversity of microorganisms may be exploited by searching the strains from the natural environment, which are capable of producing the product of interest. Useful microorganisms performing desired reactions or producing desired products are the unique subsets of all the microorganisms that are available. Microorganism isolated from nature exhibits cell growth as their main physiological property. However, in order to survive in special environments to which they are adapted, then evolve and acquire special characteristics that may be exploited for commercial applications. Some of the commonly used conventional methods for screening of useful microorganisms include the following: (1) isolation of microorganisms in the neighborhood of habitats with enhanced concentration of the substrates, (2) selection of strains based on taxonomic closeness to prior successful strains, and/or (3) enrichment culture. Such screening strategies are empirical, labor intensive, and have low success rate. Screening becomes more difficult in the absence of suitable selection criterion such as antibiotic resistance or production of any specific distinguishable characteristic (such as pigment production). Screening becomes more tedious if one has to search for an organism carrying out a particular type of reaction to give a particular product without any observable property. There are innumerable possibilities with no guaranteed assurance of obtaining the desired organism. In addition, every organism has different culture conditions that may not be provided during initial isolation and screening experiments (due to lack of knowledge of specific growth conditions), and this may lead to non-cultivability of some of the organisms.

Since extensive databases of suitable microorganism are available, it is possible to conceive

that a preliminary search may be possible to target a set of candidate organisms (Malviya and Gomes 2009). This preliminary theoretical analysis based on data-mining techniques may reduce time, effort, and cost of isolating new microorganisms with unknown potential for solid-state bioprocesses. Later successful candidate microorganisms can be improved via modern molecular protocols. Here again, it is possible to carry out a systems based (systems biology) analysis whereby key genes and enzymes can be determined and targeted.

Concluding Comments

Research in solid-state bioprocesses has gained renewed vigor. The trend now is to bring in quantitative analysis and accuracy in characterizing solid-state bioprocesses. Various aspects of this field have opened up a series of research issues that must be addressed to advance this field into the next stage. The progress in the 1990s has been tremendous and has brought solid-state bioprocesses to a threshold of new milestones. It may be expected the coming decade of research will address and solve many bottleneck problems presented in this chapter.

References

Agblevor FA, Evans RJ, Johnson KD (1994) Volumetric-beam mass-spectrometric analysis of lignocellulosic materials I. Herbaceous biomass. J Anal Appl Pyrolysis 30:125–144

Ajila CM, Brar SK, Verma M, Tyagi RD, Godbout S, Valéro JR (2012) Bio-processing of agro byproducts to animal feed. Crit Rev Biotechnol 32(4):382–400

Ashley VM, Mitchell DA, Howes T (1999) Evaluating strategies for overcoming overheating problems during solid-state fermentation in packed bed bioreactors. Biochem Eng J 3:141–150

Basu S, Gaur R, Gomes J, Sreekrishnan TR, Bisaria VS (2002) Effect of seed culture on solid-state bioconversion of wheat straw by *Phanerochaete chrysosporium* to animal feed. J Biosci Bioeng 93:25–30

Bhatnagar A, Kumar S, Gomes J (2008) Operating conditions of a 200 l staged vertical reactor or bioconversion of wheat straw by *Phanerochaete chrysosporium*. Bioresour Technol 99:6917–6927

Chiesa S, Gnansounou E (2011) Protein extraction from biomass in a bioethanol refinery – Possible dietary applications: use as animal feed and potential extension to human consumption. Bioresour Technol 102(2):427–436

da Costa Souza L, Chundawat SPS, Balan V, Dale BE (2009) 'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies. Curr Opin Biotechnol 20:339–347

Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci 5(6):578–595

Domínguez A, Rivela I, Rodríguez Couto S, Sanromán MA (2001) Design of a new rotating drum bioreactor for ligninolytic enzyme production by *Phanerochaete chrysosporium* grown on an inert support. Process Biochem 37:549–554

Dupont A-L, Mortha G (2004) Comparative evaluation of size-exclusion chromatography and viscometry for the characterization of cellulose. J Chromatgr A 1026:129–141

Durand A, Renaud R, Almanza S, Maratray J, Diez M, Desgranges C (1993) Solid-state fermentation reactors: from lab scale to pilot plant. Biotechnol Adv 11:591–597

Gamarra NN, Villena GK, Gutiérrez-Correa M (2010) Cellulase production by Aspergillus niger in biofilm, solid-state, and submerged fermentations. Appl Microbiol Biotechnol 87:545–551

Goff BM, Murphy PT, Moore KJ (2012) Comparison of common lignin methods and modifications on forage and lignocellulosic biomass materials. J Sci Food Agric 92:751–758

Gomes J, Sreekrishnan TR, Bisaria VS (2006) Indian Patent 1235/DEL/2006

Hardin MT, Mitchell DA, Howes T (2000) An approach to designing rotating drum bioreactors for solid-state fermentation on the basis of dimensionless design factors. Biotechnol Bioeng 67:274–282

Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol 2:602–619

Kumar S, Gomes J (2008) Performance evaluation of reactors designed for bioconversion of wheat straw to animal feed. Anim Feed Sci Technol 144:149–166

Malaviya A, Gomes J (2009) Rapid screening and isolation of a fungus for sitosterol to androstenedione biotransformation. Appl Biochem Biotechnol 158:374–386

Mitchell DA, Krieger N, Stuart DM, Pandey A (2000) New developments in solid-state fermentation II. Rational approaches to design, operation and scale-up of bioreactors. Process Biochem 35:1211–1225

Morrison IM (1972) A semi-micro method for the determination of lignin and its use in predicting the digestibility of forage crops. J Sci Food Agric 23:455–463

Nagel FJ, Tramper J, Bakker MS, Rinzema A (2001a) Temperature control in a continuously mixed bioreactor for solid-state fermentation. Biotechnol Bioeng 72:219–230

Nagel FJ, Tramper J, Bakker MS, Rinzema A (2001b) Model for on-line moisture-content control during solid-state fermentation. Biotechnol Bioeng 72:231–243

Nayak R, Gomes J (2006) Sequential adaptive network assisted feed forward control for maximum biomass production in yeast fermentation. WSEAS Trans Syst 5(7):1654–1661

Ohra-aho T, Tenkanen M, Tamminen T (2005) Direct analysis of lignin and lignin-like components from softwood kraft pulp by Py-GCMS techniques. J Anal Appl Pyrolysis 74:123–128

Prado FC, Vandenberghe LPS, Lisboa CJ, Pandey A, Soccol CR (2004) Relation between citric acid production and respiration rate of *Aspergillus niger* in solid-state fermentation. Eng Life Sci 4:179–186

Raghavrao KSMS, Ranganathan TV, Karanth NG (2003) Some engineering aspects of solid-state fermentation. Biochem Eng J 13:127–135

Rahardjo YS, Tramper J, Rinjema A (2006) Modeling conversion and transport phenomena in solid-state fermentation: a review and perspectives. Biotechnol Adv 24:161–179

Raimbault M (1998) General and microbiological aspects of solid substrate fermentation. EJB Electron J Biotechnol 1:21–32

Rodríguez Couto S, Rivela I, Muñoz MR, Sanromán A (2000) Ligninolytic enzyme production and the ability of decolourisation of Poly R-478 in packed-bed bioreactors by *Phanerochaete chrysosporium*. Bioprocess Eng 23:287–293

Sahoo B, Sarawat ML, Haque N, Khan MY (2002) Influence of chemical treatment of wheat straw on carbon, nitrogen and energy balance in sheep. Small Rumin Res 44:201–206

Scarlata CJ, Hyman DA (2010) Development and validation of a fast high pressure liquid chromatography method for the analysis of lignocellulosics biomass hydrolysis and fermentation products. J Chromatogr A 1217:2082–2087

Shrivastava B, Thakur S, Khasa Y, Gupte A, Puniya A, Kuhad R (2011) White-rot fungal conversion of wheat straw to energy rich cattle feed. Biodegradation 22(4):823–831

Singhania RR, Patel AK, Scoccol CR, Pandey A (2009) Recent advances in solid-state fermentation. Biochem Eng J 44:13–18

Stuart DM, Mitchell DA, Johns MR, Litster JD (1999) Solid-state fermentation in rotating drum bioreactors: operating variables affect performance through their effects on transport phenomena. Biotechnol Bioeng 63:383–391

Suryanarayan S, Mazumdar K (2001) US Patent 6,197,573. B1

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Biochem 32:420–424

Valera HR, Gomes J, Lakshmi S, Gururaja R, Suryanarayan S, Kumar D (2005) Lovastatin production by solid-state fermentation using Aspergillus flavipes. Enzyme Microbiol Technol 37:521–526

Villas-Bôas SG, Esposito E, Mitchell DA (2002) Microbial conversion of lignocellulosic residues for production of animal feeds. Anim Feed Sci Technol 98:1–12

Rhizobacteria in Management of Agroecosystem

3

Devendra Kumar Choudhary and B.N. Johri

Abstract

Agriculture has a long history of research targeted at understanding how to improve the effectiveness of root symbionts, viz., rhizobia and mycorrhiza. A promising approach has been employed to understand how natural selection regulates changes in mutualistic interactions. A descriptive knowledge of basic evolutionary processes can be employed to develop agricultural management practices that favor the most effective symbionts. Mutually beneficial interactions between plant and associated rhizospheric microorganisms are ubiquitous which is important for ecosystem functioning. Plant-mediated mineralization for nutrient acquisition in agroecosystem would reduce the potential for nutrient losses because of tight coupling between net mineralization of N and P and plant uptake in the rhizosphere. Microorganisms and their products in the rhizosphere react to the many metabolites that are released by plant roots in a variety of positive, negative, and neutral ways. Such interactions can influence plant growth and development, change nutrient dynamics, and alter plant's susceptibility to biotic and abiotic stresses. This benefit can either persist or lost in well-fertilized agricultural soils where nutrients are readily available to plants and symbionts that reduce growth.

Keywords

Agroecosystem • Rhizosphere • Rhizobacteria • Plant growth-promoting bacteria • Symbionts

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Introduction

Sustainable agriculture involves designing farm system employing nature as a model. In most natural ecosystem, the greater the diversity, the more resistant an ecosystem to change and is better able to recover from disturbances. In an agricultural

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ecosystem or the so-called agroecosystems (AESs), disturbance is much more frequent, regular, and intense. The ecological concepts of disturbance and their recovery through succession play an important role in AESs management. AESs are undergoing disturbances in the form of cultivation, soil preparation, sowing, planting, irrigation, fertilizer application, pest management, pruning, harvesting, and burning. AESs are limited in earliest stages of succession when disturbance is frequent, widespread, and intense as it is in conventional agriculture.

The diversity and intensity of AESs in developing (Kassam et al. 2009) and developed (Izaurralde et al. 2003) countries have been changing over time in response to a number of interacting biophysical and social factors at the local, regional, and global levels. The impact of increased spatiotemporal climate variability on AESs is likely to be intensified by climate change, which will disrupt many ecosystem functions, altering their capacity to provide goods and services and rendering them more susceptible to degradation (IPCC 2007; Friend 2010). In addition, the security of food supply to an increasing world population has turned into a pressing issue worldwide (Friend 2010). Sustainable food production can be achieved by avoiding excessive disturbance and allowing successional processes to generate greater AESs stability. One can enhance the ability of AESs to maintain both fertility and productivity through appropriate management of disturbance and recovery.

Plant productivity is often limited by soil nutrient availability and the interface between living roots and soils, i.e., rhizosphere, which is a central commodity of exchange where organic C flux from root fuels and microbial decomposers that can provide nutrients available to roots. It is virtually impossible to investigate the intricacies of potential rhizosphere interaction in every environmental condition by virtue of tremendous diversity of soil microbes, soil fauna, and plants. An understanding of controls over belowground function constitutes an important challenge as natural and AESs around the globe are exposed to anthropogenic pressures (Pregitzer et al. 2006). In addition, the physicochemical and structural properties of soils including development have been strongly affected by the action of rhizosphere over consecutive evolutionary time frame, and the evolution of true plant roots along with their extension deep into substrate is considerably hypothesized to have led to a revolution in planetary C and water cycling that reflects on the biogeochemical functions of the rhizosphere on Earth today (Beerling and Berner 2005; Richter et al. 2006).

Understanding the complex microbial community in the rhizosphere environment has proven to be a challenging task because of the vast diversity and the enormity of the population inhabiting this unique habitat. Extensive studies have investigated perturbation of microbial community equilibrium population by changes in environmental conditions and soil management practices (Sun et al. 2004). It has long been recognized that the activity of soil microorganisms plays an intrinsic role in residue decomposition, nutrient cycling, and crop production. Any shift in microbial community structure can be reflected in implementation of various land use and management systems that lead to development of best management practices for an AES (Peacock et al. 2001).

In subsistence AESs, crop yields are directly dependent on the inherent soil fertility and on microbial processes that govern the mineralization and mobilization of nutrients required for plant growth. In addition, the impact of different crop species that are used in various combinations is likely to be an important factor in determining the structure of plant beneficial microbial communities that function in nutrient cycling, the production of plant growth hormones, and suppression of root diseases (Alvey et al. 2003). Microorganisms represent a substantial portion of the standing biomass in terrestrial ecosystem and contribute in regulation of C sequestration, N availability and losses, and P dynamics. The size and physiological state of the standing microbial biomass is influenced by management practices including rotational diversity, tillage, and the quality and quantity of C inputs to the soils (Fliessbach and Mader 2000).

A key premise in AES preservation that has been globally accepted is derived from the concept of sustainability and sustainable ecosystem that can be achieved by means of the rationale use of natural resources. In AES, sustainability is dependent on biological balance in the soils that is governed by the activity of microbial communities. Soil microbial populations are immersed in a framework of interactions known to affect plant fitness and soil quality; thereby, the stability and productivity of both AES and natural ecosystem are enhanced (Barea et al. 2005). The global necessity to increase agricultural productivity from steadily decreasing and degrading land resources base has placed significant strain on the fragile agroecosystems. Therefore, it is necessary to adopt strategies to maintain and improve agricultural productivity employing high-input practices. Improvement in agricultural sustainability requires optimal use and management of soil fertility and soil physical properties and relies on soil biological processes and soil biodiversity. It is necessary to understand perspectives of microbial diversity in agricultural context that is important and useful to arrive at measures that can act as indicators of soil quality and plant productivity (Tilak et al. 2005).

In this chapter, particular stress has been given on the role of microbial diversity, including rhizospheric action, in securing crop protection and soil fertility because of their persistence upon the maintenance of biological integrity and diversity together with management in AESs.

Nutrients in Agroecosystems: Management Paradigm

Sustainability of agroecosystems (AESs) cannot be understood using any single dimension or criterion (Belcher et al. 2004), and the feedback between crop production and each of crop rotation, soil characteristics, and several management practices is an important driver of AES sustainability (Hobbs et al. 2008). The biophysical characteristics of any AES are critical determinants of its overall performance and sustainability (Belcher et al. 2004). There are unintended consequences of modern agriculture that extend well beyond agricultural landscapes. Biogeochemical cycles have been profoundly altered at multiple scales and exceed soil loss over soil formation. Nitrogen (N) and phosphorus (P) are the two most important nutrients that limit biological production and are the most extensively applied nutrients in managed agroecosystem in the form of soluble inorganic fertilizers (F_i). Nutrient enrichment reflects detrimental effects in agroecosystem wherein global N and P fluxes are projected to increase substantially with concomitant inorganic fertilizers production capacity (Galloway 2000). Therefore, a new approach to nutrient supply in intensively managed ecosystem is required to control their process of eutrophication wherein an integrated nutrient management (INM) strategy based on ecological concepts has been applied. As a result, nutrient management research continues to emphasize improved delivery of F_i to the root zone during the period of crop uptake through several modification, viz., banding, fertigation (method of applying fertilizers, soil amendments, and other water soluble products required by the plants during its growth stages through drip/sprinkler irrigation system), and split fertilizer applications (Cassman et al. 2002).

Functioning and Assessment of Biodiversity in Agroecosystem: Ecological Role

Biodiversity has become a central concept in agronomical research that is considered being a complex entity which can be spread over several levels, viz., genes, species, ecosystem, and ecological processes, that relate to three main functions: (1) patrimonial (concerns conservation of the landscape aesthetic and threatened species) function, (2) agronomical function, and (3) ecological function. The patrimonial function involves conservation of the landscape aesthetic and threatened species. The biodiversity function concomitantly with agricultural activities describes resistance to biotic and abiotic stresses and the production of cultivated ecosystem.

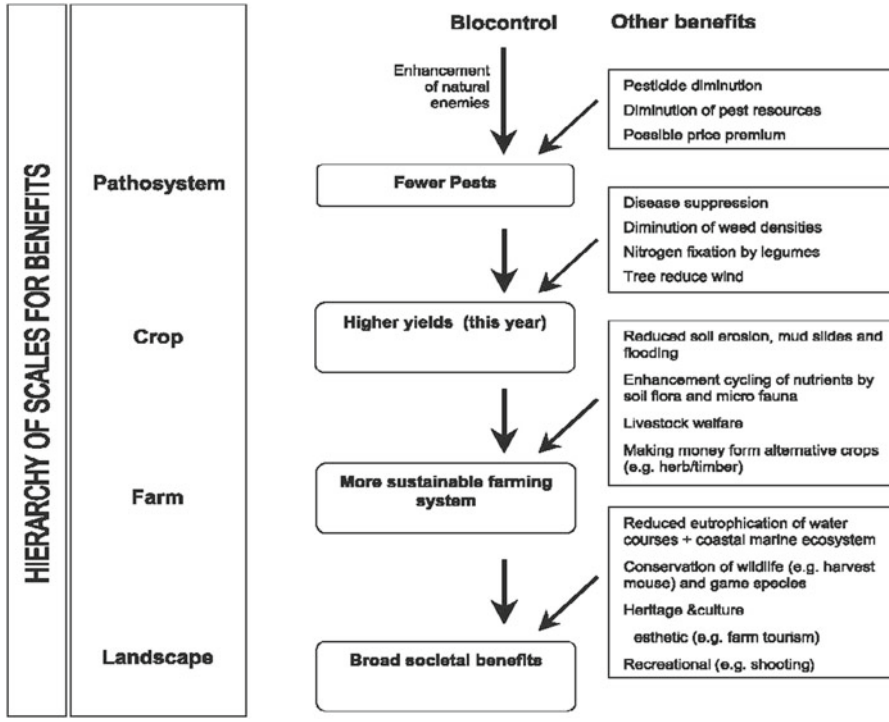

Fig. 3.1 The hierarchy of scale for potential benefits of multifunctional agricultural biodiversity (Clergue et al. 2005; Reproduced with permission) (Yes! permission for this figure was taken and submitted to editor with soft and hard copy of chapter when book was proposed for publication in the year 2006 or 2007)

Biodiversity is also involved in ecological functioning through the existence of special habitats with particular species (Clergue et al. 2005). Gurr et al. (2003) described benefits of biodiversity for agricultural production, e.g., pest management that favors enhancement of natural enemies. These workers have proposed a hierarchy of biodiversity benefits based on the different scales of biodiversity (Fig. 3.1).

Plant Diversity Employed to Restore Ecosystem Function

Efficient application of plant diversity to restore agroecosystem functions reflects a more sophisticated approach than simply reinstating traditional rotations. Plants and their associated microbes regulate myriad of processes that ultimately control ecosystem fluxes of C, N, and P (Eviner and Chapin 2001). The capacity of plant species to contribute to ecosystem processes will help restore desired agroecosystem functions and increase yields in systems where fertilizers are currently under applied. A significant contribution of plant species effects has been reported for decomposition dynamics and mineralization of N and P, aggregate formation, ability to access nutrients, e.g., Ca, Mg, and P from mineral sources, and microbial community composition and function (Kent and Triplett 2002; Cheng et al. 2003; Vance et al. 2003). An appropriate use of cover crops instead of bare fallows has been considered as foremost priority for nutrient management program. It has been reported that cover cropping reduced nitrate leaching by an average of 70% without incurring any sacrifice in yield compared to conventional rotations where gaps between crops were maintained as bare fallows.

Restoration of Agroecosystem Function Through Plant-Microbe Interactions

Agriculture has a long history of research targeted at understanding how to improve the effectiveness of root symbionts, viz., rhizobia and mycorrhiza. A promising approach has been employed to understand how natural selection regulates changes in mutualistic interactions (Kiers et al. 2002; Denison et al. 2003b). A descriptive knowledge of basic evolutionary processes can be employed to develop agricultural management practices that favor the most effective symbionts. Mutually beneficial interactions between plant and associated rhizospheric microorganisms are ubiquitous which is important for ecosystem functioning. Symbiotic nitrogen fixation by bacteria, e.g., *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium*, and *Azorhizobium* spp. that collectively known as rhizobia or by *Frankia* spp., is the major N input to many natural and agricultural ecosystems in the root nodules of legumes or actinorhizal plants, respectively. In addition, mycorrhizal fungi supply their host plants with mineral nutrients, viz., P and other benefits. Several rhizospheric microorganisms cause severe infection to roots and so-called root pathogens that can be suppressed by *Pseudomonas fluorescens* after colonization of the roots, thereby improving plant health (Denison et al. 2003a, b). Plant-mediated mineralization for nutrient acquisition in agroecosystem would reduce the potential for nutrient losses because of tight coupling between net mineralization of N and P and plant uptake in the rhizosphere. Microorganisms and their products in the rhizosphere react to the many metabolites that are released by plant roots in a variety of positive, negative, and neutral ways. Such interactions can influence plant growth and development, change nutrient dynamics, and alter plant's susceptibility to disease and abiotic stresses. Overall the general rhizosphere effect could help the plant by maintaining the recycling of nutrients through the production of hormones that help provide resistance to microbial diseases and to aid tolerance to toxic compounds. This benefit can either persist or lost in well-fertilized agricultural soils where nutrients are readily available to plants and symbionts that reduce growth (Morgan et al. 2005).

Microbial-Mediated Processes Benefiting Sustainable Agroecosystem Development

Microorganisms represent a substantial portion of the standing biomass in terrestrial ecosystem that contributes to the regulation of C sequestration, N availability and losses, and P dynamics. Microbial biomass P turnover is rapid which is approximately twice as fast as C, suggesting the potential for microbial P pools to support plant P requirements (Kouno et al. 2002). Heterotrophs in soils with larger plant species diversity convert a greater proportion of metabolized C to biomass (Aoyama et al. 2000). The intentional management of the microbial community to enhance N retention in soils makes it possible to characterize abundance and activity of microbial functional groups. Denitrifiers in agricultural soils are more sensitive to O_2 levels that produce a greater proportion of N_2O compared to denitrifiers recovered from an early successional plant community. The rate of denitrification and the proportion of N_2O to N_2 produced affect the denitrifier community composition (Cavigelli and Robertson 2001).

There is an increasing interest in understanding the cooperative activities among microbial populations because of current public concerns about the adverse effect of agrochemicals and how do they affect agroecosystem when applied in agricultural soils (Lucy et al. 2004). Two types of interactions in the rhizosphere are recognized mainly wherein one is based on dead plant material (the detritus-based interactions) and other involves living plant roots. Both types of interactions are relevant to agronomy and ecology. Microbial activity in the rhizosphere affects rooting pattern and the supply of available nutrients to plants thereby modifying the quality and quantity

of root exudates (Gryndler 2000). The specific structure and diversity of the rhizosphere bacterial community vary between plant species and over time, and the different root zones present on the same plant can support distinct bacterial communities that reflect on the qualitative and quantitative differences in root exudation (Smalla et al. 2001; Yang and Crowley 2000). In addition, functioning of bacterial communities in agroecosystem is affected by soil type that plays a key role in determining the specific dominant bacteria colonizing the rhizosphere (Marschner et al. 2001). The development of sustainable agroecosystem is, therefore, affected by the type of interactions in the rhizosphere that include (1) the cooperation between PGPR and *Rhizobium* for improving N_2 fixation, (2) microbial antagonism for the biocontrol of plant pathogen, and (3) interaction between rhizospheric microbes and AM fungi to develop functional mycorrhizosphere milieu.

Rhizosphere supports bacterial communities that stimulate growth of plants and the so-called plant growth-promoting rhizobacteria (PGPR). Such PGPR operate by a wide variety of mechanisms that include N_2 fixation, enhanced solubilization of P, and phytohormone production; among these, pseudomonads are considered an important rhizosphere organism (Vessey 2003; Lugtenberg et al. 2001). Over 80% of the terrestrial ecosystem is able to form mycorrhizal association wherein there is bidirectional flow of nutrients, i.e., carbon flows from the plant host to the fungus and mineral nutrient flow from the fungus to the plant. In addition, mycorrhizal fungi are able to provide protection to the host plant against root and shoot pathogens (Whipps 2004). Mycorrhizosphere inhabitants include intra-hyphal bacteria in ectomycorrhizal fungi and intraspore bacteria in several arbuscular fungi. Several mycorrhizosphere bacteria can promote mycorrhiza formation wherein a variety of Gram-positive and Gram-negative strains are involved (Bertaux et al. 2003). In natural agroecosystem, the diversity of AM fungi is considered as a key contributor to the diversity and productivity of plant community. The composition of root-inhabiting AM community shows seasonal variation within individual host plants, and this can change with plant maturity (Heinemeyer et al. 2004).

Many farming practices, viz., fertilizer input, cultivation, and fumigation, exert deleterious effects on communities of AM fungi that are known to be less diverse and abundant in conventional agricultural system as compared to organically managed and seminatural areas (Oehl et al. 2004). The AM-fungus communities recovered from organic practices are more beneficial for crop yield than those from intensive conventional practices, and there is availability of commercial inocula of AM fungi for use in degraded habitats and agricultural systems, but application of these products has been relatively limited to date (Gianinazzi and Vosatka 2004).

It is a well-accepted view that symbiotic legumes benefit companion and subsequent plant species in intercrop and rotation system. Rhizobia (species of *Rhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Sinorhizobium,* and *Mesorhizobium*) produce chemical molecules that influence plant development including phytohormones, lipo-chitooligosaccharides, Nod factors, lumichrome, riboflavin, and H_2 evolved by nitrogenase. Nod factor stimulates seed emergence, promotes plant growth, and increases grain yield when they reside in the soil. Rhizobia are known to suppress the population of soil pathogens in agricultural and natural ecosystem, viz., a strain of *Bradyrhizobium japonicum* can cause up to a 75% decrease in sporulation of *Phytophthora megasperma,* 65% in *Pythium ultimum*, 47% in *Fusarium oxysporum*, and 35% in *Ascochyta imperfecta*. From an agricultural point of view, the most significant interactions are those of the Fabaceae-*Rhizobium* spp./*Bradyrhizobium* spp. root nodule symbioses (Squartini 2003). Recent work on root nodule bacteria has demonstrated that this interaction is not restricted to *Rhizobium*/*Bradyrhizobium* but includes N_2-fixing strains of *Ralstonia, Burkholderia*, and *Methylobacterium* that have been recovered from the nodules of several tropical Fabaceae (Dakora 2003). The plant-bacterial association has been commercially exploited wherein seed and soil inoculants of rhizobia are employed for many crops that include soybean, bean, peanut, and clover (Deaker et al. 2004).

Considerable research efforts have been placed globally to exploit the potential of fluorescent pseudomonads (FLPs) in maintenance of soil health and as crop protectants wherein antifungal compound 2, 4-diacetylphloroglucinol (DAPG) produced by FLPs did not influence AM fungi in wheat rhizosphere (Gaur et al. 2004). In addition, these workers reported that 50–60% of FLPs recovered from rhizosphere, and endorhizosphere of wheat grown in Indo-Gangetic plains was antagonistic toward *Helminthosporium sativum*. Johri (2001) emphasized that field trials of pseudomonad strain GRP_3 enhanced crop yield increase from 5.6 to 18%.

Tillage Effects and Resilience of Agroecosystem

Tillage is considered the most effective farm activity for the purpose of developing a desired soil structure. It improves the physical conditions of soil and favors the rooting characteristics of plants, which lead to an enhanced nutrient uptake and better yield of crops. Weeds are probably the most ever-present class of crop pests and on the odd occasion cause massive crop failures over vast areas. They reduce the crop yield and deteriorate the quality of produce and hence reduce the market value of the turnout (Arif et al. 2006). They use the soil fertility, available moisture, and nutrients and compete for space and light with crop plant, which result in yield reduction (Khan et al. 2002, 2004). If left uncontrolled, the weeds in many fields are capable of reducing yields by more than 80% (Karlen et al. 2002).

The composition of weed communities is greatly affected by tillage, but it is difficult to control weeds in reduced tillage (RT). The disadvantages of RT are infestations by several annual and perennial species and rapid increase of the seed bank near the soil surface. Hence, occasional or rotational use of RT may be a practical way to adopt RT into conventional tillage (CT) systems. Changes between tillage practices, from CT to RT and vice versa, were effective in suppressing weed growth and preventing seed accumulation (Nakamoto et al. 2006).

Arbuscular mycorrhizal fungi (AMF) are obligate symbiont fungi, which have a wide distribution in the terrestrial ecosystems and in a vast diversity of climate and soil types, forming symbiotic associations with the majority of plants (Jeffries et al. 2003). In an ecosystem context, the AMF activity affects the carbon dynamics by different mechanisms (Zhu and Miller 2003), among them, the protection of organic matter into soil aggregates by means of the AMF mycelium and the production of glomalin. This compound has been operationally defined and extracted from soil as glomalin-related soil protein GRSP (Rillig 2004) of a proteic nature (Gadkar et al. 2006) and highly recalcitrance compound (Driver et al. 2005). Previous studies have linked the GRSP content with the aggregate stability (Wright et al. 2007) and soil C accumulation (Treseder and Turner 2007).

Soil management, in special, the tillage systems, affects all soil properties, including AMF activity, diversity (Alguacil et al. 2008; Borie et al. 2008; Cornejo et al. 2009), and GRSP production (Wright et al. 2007), being important factors controlling organic C storage in soils. They may also change the relative importance of different mechanisms of soil organic matter (SOM) stabilization (John et al. 2005). On the other hand, no-tillage agriculture, which returns organic residues to soil, can produce a positive effect on soil characteristics. Thus, the study of AMF role and glomalin is important for evidencing soil C dynamics and its contribution to the stabilization of soil C in the agricultural systems with the goal of improving the sustainability of agroecosystems.

Biodiversity Benefits for Agroecosystem

While species extinction is a matter of increasing concern, changes in biodiversity in the world's agricultural landscapes have largely escaped attention. Yet, agriculture is fundamentally linked to biodiversity. "Biological diversity" or biodiversity has formed the basis for human food production systems for millennia and plays an important role in the provisioning services, i.e.,

production of foods, fuels, and fibers, that agriculture supplies. Biodiversity in agriculture also provides cultural services that form key elements of the agricultural knowledge base and defines spiritual, religious, and aesthetic values for human societies. In a wider context, biodiversity serves important functions that enhance the environmental resource base upon which agriculture depends, e.g., regulating and supporting services such as water purification, nutrient cycling, and soil formation.

Soil biota regulates many ecological processes, viz., litter decomposition, nutrient cycling, pathogen control, and mineral weathering. The processes of decomposition, immobilization, and mineralization release nutrients in soil that affect plant growth. In addition, symbiotic association with mycorrhizal fungi enhances nutrient (P) availability and increases plant water uptake. The biodiversity of soil organisms and their abundance are involved with processes that affect soil structure. Soil structuring increases the growth of plant, root anchorage, and fluid circulation (air and soil solution). The farming practices (fertilization, pesticides, and tillage) affect the population size and the dynamics of several group, viz., microbes, protozoa, vascular plant, nematodes, arthropods, annelids, and vertebrates (Smeding and de Snoo 2003). In agricultural systems, biodiversity performs ecosystem services beyond production of food, fiber, fuel, and income that includes nutrient recycling, control of local microclimate, regulation of the abundance of undesirable organisms, and detoxification of noxious chemicals. These renewal processes and ecosystem services are largely biological, and therefore, their persistence depends upon maintenance of biological diversity. The economic and environmental costs can be quite significant when these natural services are lost because of biological simplification.

There are two distinct components of biodiversity recognized in an agroecosystem wherein the first component, which is the so-called planned biodiversity, is the biodiversity associated with the crops and livestock that are included in the agroecosystem by the farmer and vary depending on the management inputs and crop spatial/temporal arrangements, whereas the second component, which is called associated biodiversity, includes all soil flora and fauna, herbivores, carnivores, and decomposers that colonize the agroecosystem from surrounding environment and that will thrive in the agroecosystem depending on its management and structure. There are many agricultural practices and designs that have the potential to enhance biodiversity, whereas other practices negatively affect it (Fig. 3.2).

This is required for the management practices in order to enhance the kind of biodiversity that cannot only subsidize the sustainability of agroecosystem by providing ecological services. Thus, the main emphasis in agroecology is to exploit the complementarities and synergisms that result from combinations of crops, trees, and animals in spatial and temporal arrangements. Besides, we have to adopt those agricultural practices that increase the abundance and diversity of above- and belowground organisms thereby providing key ecological services to agroecosystems.

Epilogue

Agroecosystems and agrobiodiversity contribute to sustainable livelihood securities at the local, national, and global levels. They provide a range of goods and services including food, fodder, climate change mitigation, biodiversity conservation, and water quality options. Farmers and farming communities have a significant role to play in the preservation and conservation of these resources and ecosystems. The role of agriculture in the provision of ecosystem services depends, however, on the incentives available to it. At present incentives are designed to pay for the goods rather than the services provided by agricultural ecosystems. Payments for ecosystem services interventions often do not reflect correctly.

We are in an era of rapid ecological, social, and economic change, and the pace of this change will accelerate during the next several decades. The world's population of 6.3 billion people is projected to grow to 7.5 billion by the year 2020 and to 9 billion by 2050 (Tilman et al. 2002).

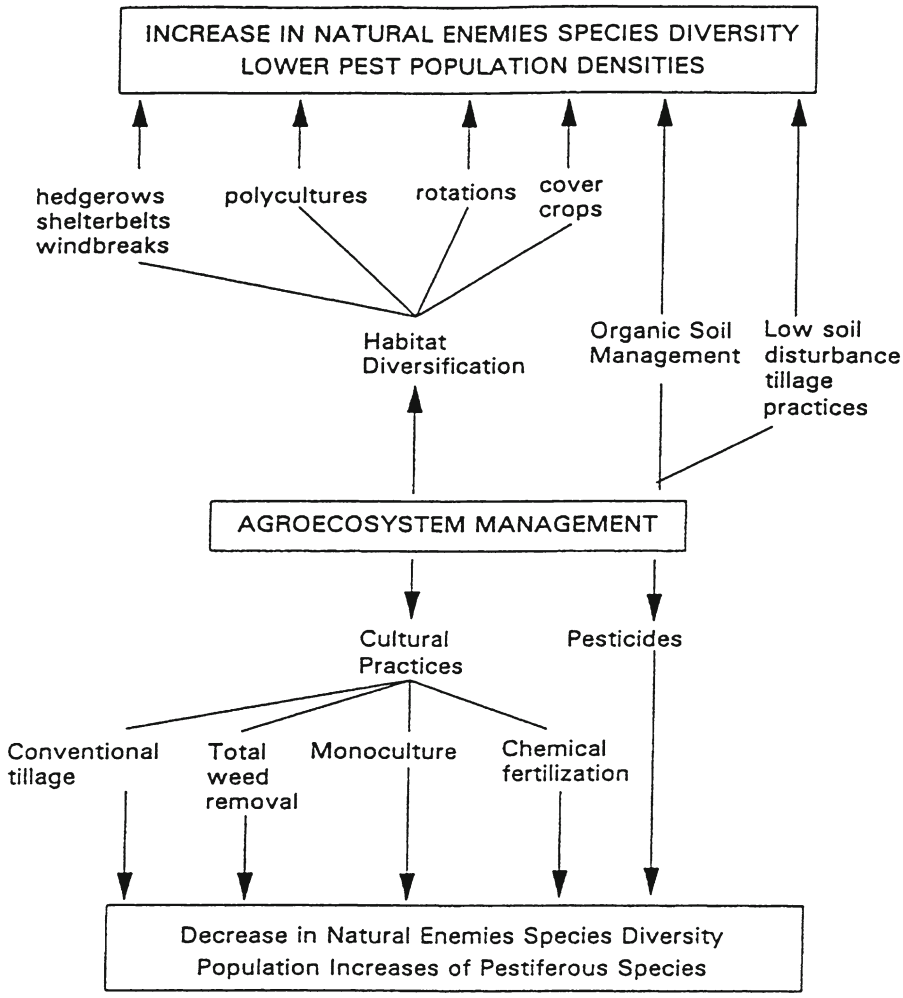

Fig. 3.2 The effects of agroecosystem management and associated cultural practices on the biodiversity of natural enemies and the abundance of insect pests (Altieri 1999; Reproduced with permission) (Permission for this figure was taken and submitted to editor with soft and hard copy of chapter when book was proposed for publication in the year 2006 or 2007)

By 2050, food consumption must double to meet human needs. To meet this increasing demand for food, production systems are expected to become increasingly dependent on inputs of fertilizers, pesticides, and water. Irrigated lands will likely increase by 1.3-fold by 2020 and 1.9-fold by 2050. Pasture lands are also increasing, with an expected doubling in area by 2050. In 50 years, global agricultural land area is projected to increase by 18%, with a loss of 10^9 ha of natural, wild land ecosystems.

Biologically, there is much potential for increasing the utilization of agrobiodiversity for sustainable agriculture. The agricultural sciences have shown for decades that plant breeding can introduce genes to increase the quality of agricultural production, though recent advances with genetically modified organisms (GMOs) are very controversial. Crop diversity can clearly enhance nutrient use efficiency, and a diverse soil community may govern tighter nutrient cycling, control pests and diseases, and improve soil structure ("let the soil work for us"). Ecological research has shown that biodiversity can increase the productivity of ecosystems. The structure of agricultural landscapes, i.e., mosaics of agricultural and

nonagricultural ecosystems, now is recognized as important for the utilization and conservation of both on-farm and off-farm biodiversity. Economists have shown how investment in agrobiodiversity reduces both the environmental and market risks faced by agricultural producers, and so enhances well-being.

Research and institutional and policy settings for technological innovations in agriculture are, however, changing rapidly. Innovations now require plurality of systems and multiple sources (Janssen and Braunschweigg 2003). Linking technological progress with institutional and market changes is the need of the hour. Some key recent conclusions emanating from technological innovations include that genetic improvements are successful, but not everywhere; there is a need for management and system technologies to complement genetic improvement; more investments are needed into research and development; the use of available technologies such as information and communication technology has still not permeated some parts of the world to improve the efficiency of agricultural systems; and innovative partnerships are key. Sharing of data and information and infrastructural developments and deployment will help to ensure better reach and impacts of available innovations.

The search for self-sustaining, low-input, diversified, and energy-deficient agricultural system is now a major concern of many researchers, farmers, and policy makers. It is necessary to restore functional biodiversity of the agricultural landscape in sustainable agriculture. Now there are different options available to diversify cropping system depending on the monoculture system. Rotation and multiple cropping systems are effective management strategies considered for monocultures. There is economic and ecological sustainability of the agroecosystem which is a result of agroecological design with proposed management system that can be improved employing available biodiversity and with the existing environmental and socioeconomic condition.

Plant health is intimately linked to the health of the agroecosystem in which the plant flourishes. Instead of rotating and diversifying crops regularly and promoting soil organic matter buildup, farmers choose crops that require less labor or receive higher prices, thus disrupting the self-regulatory mechanism between beneficial and detrimental organisms above- and belowground. Inappropriate agricultural practices, such as extensive mechanical tilling, also change the physical and chemical nature of the soil and hence alter the sound ecological balance of different soil organisms, affecting long-term agricultural productivity and sustainability.

Compared to the integrated management of specific pest organisms above ground, relatively little research has been conducted on the integrated management of soil health as an approach to control soilborne pests and diseases and to improve nutrient availability and water uptake for optimum crop growth. Soil ecosystems are among the most complex of all terrestrial communities, and the role of soil biota in maintaining plant health is not fully understood. Improved resilience together with increased deployment of biodiversity is necessary to achieve the goals of sustainability and productivity. Agroecosystems have to be managed in ways that conserve and enhance functional agrobiodiversity, including abundance of soil biota and diversity, even with further intensification of agricultural production. Diverse soil communities will not only help to prevent losses through soilborne pests but will also increase the rate of decomposition of organic matter and toxic compounds and improve nutrient recycling and soil structure. Increasing the resilience of agroecosystems helps farmers to deal better with the impact of climate change.

Plant growth in agricultural soils is influenced by a myriad of abiotic and biotic factors. While growers routinely use physical and chemical approaches to manage the soil environment to improve crop yields, the application of microbial products for this purpose is less common. An exception to this is the use of rhizobial inoculants for legumes to ensure efficient nitrogen fixation. The region around the root, the rhizosphere, is relatively rich in nutrients, due to the loss of as much as 40% of plant photosynthates from the roots. Consequently, the rhizosphere supports large and active microbial populations capable of

exerting beneficial, neutral, or detrimental effects on plant growth. The importance of rhizosphere microbial populations for maintenance of root health, nutrient uptake, and tolerance of environmental stress is now recognized. These beneficial microorganisms can be a significant component of management practices to achieve the attainable yield, which has been defined as crop yield limited only by the natural physical environment of the crop and its innate genetic potential.

The rhizosphere or the zone of influence around roots harbors a multitude of microorganisms that are affected by both abiotic and biotic stresses. Among these are the dominant rhizobacteria that prefer living in close vicinity to the root or on its surface and play a crucial role in soil health and plant growth. Both free-living and symbiotic bacteria are involved in such specific ecological niches and help in plant matter degradation, nutrient mobilization, and biocontrol of plant disease. While the rhizosphere as a domain of fierce microbial activity has been studied for over a century, the availability of modern tools in microbial ecology has now permitted the study of microbial communities associated with plant growth and development, in situ localization of important forms, as well as the monitoring of introduced bacteria as they spread in the soil and root environment. This interest is linked to environmental concerns for reduced use of chemicals for disease control as well as an appreciation for utilization of biologicals and organics in agriculture.

Indian researchers have studied the diversity of rhizobacteria in a variety of plants, cereals, legumes, and others along with assessment of their functionality based on the release of enzymes (soil dehydrogenase, phosphatase, nitrogenase, etc.), metabolites (siderophores, antifungals, HCN, etc.), and growth promoters (IAA, ethylene) and as inducers of systemic disease resistance (ISR). Based on such primary screening protocols, effective rhizobacteria have been field tested with success stories from various agroecological zones of the country, as reflected in the control of root- and soilborne diseases, improved soil health, and increased crop yields. Several commercial formulations, mostly based on dry powder (charcoal, lignite, farmyard manure, etc.), have been prepared and field tested; however, problems of appropriate shelf life and cell viability are still to be solved. Also, inherent in such low-cost technologies are the problems of variability in field performance and successful establishment of introduced inoculants in the root zone. In addition, most products available in the market are not properly monitored for quality before they reach the farmer. As a consequence, the acceptance of rhizobacterial formulations in the country is limited. However, several laboratories have now developed protocols for the rapid characterization of effective isolates based on molecular fingerprinting and other similar tools. Also, the use of molecular markers (*gus, lux, gfp*, etc.) makes it easy to monitor introduced inoculants in situ in soil and rhizosphere environments.

The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field. The potential environmental benefits of this approach, leading to a reduction in the use of agricultural chemicals and the fit with sustainable management practices, are driving this technology. Recent progress in our understanding of the biological interactions that occur in the rhizosphere and of the practical requirements for inoculant formulation and delivery should increase the technology reliability in the field and facilitate its commercial development. The government initiative in integrated nutrient management and pest management systems has provided additional incentives to relate rhizobacterial science to other ongoing activities so that the benefit of this research leads to technologies that are environmentally and socially acceptable.

The success of soil ecosystem management does not rely simply on the choice of agricultural practices. The history of farming site affects the outcome of soil ecosystem management. An important practice that enhances soil health is the use of OM, the decomposition rate, and the products of organic material in the soil that depend on their nature and C:N ratio along with time course

of decomposition. Monitoring of the soil ecosystem prior to the cropping will help in determining the practices that are selected. Finally, the concept of soil ecosystem management is still at a developmental stage, and there is a need to have more information that must still be added to advance our understanding of soil health for agroecosystem management. Interdisciplinary approaches are necessary to broaden the application of microorganisms in ecosystem management.

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References

Alguacil MM, Lumini E, Roldan A, Salinas-Garcia JR, Bonfante P, Bianciotto V (2008) The impact of tillage practices on arbuscular mycorrhizal fungal diversity in subtropical crops. Ecol Appl 18:527–536

Altieri MA (1999) The ecological role of biodiversity in agro-ecosystems. Agric Ecosyst Environ 74:19–31

Alvey S, Yang C-H, Buerkert A, Crowley DE (2003) Cereal/legume rotation effects on rhizosphere bacterial community structure in West African soils. Biol Fertil Soils 37:73–82

Aoyama M, Angers DA, ŃDayegamiye A, Bissonnette N (2000) Metabolism of 13C-labelled glucose in aggregates from soil with manure application. Soil Biol Biochem 32:295–300

Arif M, Khan MA, Akbar H, Ali S (2006) Prospects of wheat as a dual purpose crop and its impact on weeds. Pak J Weed Sci Res 12:13–17

Barea J-M, Pozo MJ, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Beerling DJ, Berner RA (2005) Feedbacks and the coevolution of plants and atmospheric CO_2. Proc Natl Acad Sci U S A 102:1302–1305

Belcher K, Boehm M, Fulton M (2004) Agroecosystem sustainability: a system simulation model approach. Agr Syst 79:225–241

Bertaux J, Schmid M, Prevost-Boure NC, Churin JL, Hartmann A, Garbaye J, Frey Klett P (2003) *In situ* identification of intracellular bacteria related to *Paenibacillus* spp., in the mycelium of the ecto-mycorrhizal fungus *Laccaria bicolor* S238N. Appl Environ Microbiol 69:4243–4248

Borie F, Rubio R, Morales A (2008) Arbuscular mycorrhizal fungi and soil aggregation. J Soil Sci Plant Nutr 8:9–18

Cassman KG, Dobermann A, Walters DT (2002) Agroecosystems, nitrogen-use efficiency, and nitrogen management. Ambio 31:132–140

Cavigelli MA, Robertson GP (2001) Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem. Soil Biol Biochem 33:297–310

Cheng W, Johnson DW, Fu S (2003) Rhizosphere effects on decomposition: control of plant species, phenology, and fertilization. Soil Sci Soc Am J 67:1418–1427

Clergue B, Amiaud B, Pervanchon F, Lasserre-Joulin F, Plantureure S (2005) Biodiversity: function and assessment in agricultural areas. A Rev Agron Sustain Dev 25:1–15

Cornejo P, Rubio R, Borie F (2009) Mycorrhizal propagule persistence in a succession of cereals in a disturbed and undisturbed andisol fertilized with two nitrogen sources. Chil J Agric Res 69:426–434

Dakora FD (2003) Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. New Phytol 158:39–49

Deaker R, Roughley RJ, Kennedy IR (2004) Legume seed inoculation technology – a review. Soil Biol Biochem 36:1275–1288

Denison RF, Bledsoe C, Kahn M, Ó Gara F, Simms EL, Thomashow LS (2003a) Cooperation in the rhizosphere and the "free rider" problem. Ecology 84(4):838–845

Denison R-F, Kiers ET, West S-A (2003b) Darwinian agriculture: when can humus find solutions beyond the reach of natural selection? Q Rev Biol 78(2):145–168

Driver JD, Holben WE, Rillig MC (2005) Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. Soil Biol Biochem 37:101–106

Eviner VT, Chapin FS III (2001) Plant species provide vital ecosystem functions for sustainable agriculture, rangeland management and restoration. Calif Agric 55:54–59

Fliessbach A, Mader P (2000) Microbial biomass and size-density fractions differ between soils of organic and conventional agricultural systems. Soil Biol Biochem 32:757–768

Friend AD (2010) Terrestrial plant production and climate change. J Exp Bot 61:1293–1309

Gadkar V, Driver JD, Rillig MC (2006) A novel in vitro cultivation system to produce and isolate soluble factors released from hyphae of arbuscular mycorrhizal fungi. Biotechnol Lett 28:1071–1076

Galloway JN (2000) Nitrogen mobilization in Asia. Nutr Cycl Agroecosyst 57:1–12

Gaur R, Shani N, Kawaljeet, Johri BN, Rossi P, Aragno M (2004) Diacetyl Phloroglucinol-producing *Pseudomonas* do not influence AM fungi in wheat rhizosphere. Curr Sci 86:453–457

Gianinazzi S, Vosatka M (2004) Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. Can J Bot 82:1264–1271

Gryndler M (2000) Interactions of arbuscular mycorrhizal fungi with other soil organisms. In: Arbuscular mycorrhizas: physiology and functions. In: Kapulnik Y, Douds DD Jr (eds) Arbuscular mycorrhizas: physiology and functions. Kluwer Academic Publishers, Dordrecht, pp 239–262

Gurr GM, Wratten SD, Luna JM (2003) Multifunction agriculture biodiversity measures with bacterial communities. FEMS Microbiol Ecol 43:1–11

Heinemeyer A, Ridgway KP, Edwards EJ, Benham DG, Young JPW, Fitter AH (2004) Impact of soil warming and shading on colonization and community structure of arbuscular mycorrhizal fungi in roots of a native grassland community. Glob Chang Biol 10:52–64

Hobbs P, Sayer K, Gupta R (2008) The role of conservation agriculture in sustainable agriculture. Philos Trans R Soc B 363:543–555

IPCC (2007) Impacts, adaptation and vulnerability. In: Parry ML, Canziani O, Palutikof JP, van der Linden PJ, Hanson CE (eds) Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, p 976

Izaurralde R, Rosenberg N, Brown R, Thomson A (2003) Integrated assessment of Hadley Center (HadCM2) climate-change impacts on agricultural productivity and irrigation water supply in the conterminous United States Part II. Regional agricultural production in 2030 and 2095. Agr Forest Meteorol 117:97–122

Janssen W, Braunschweigg T (2003) Trends in the organization and financing of agricultural research in developed countries: implications for developing countries. ISNAR research report no. 22, International Service for National Agricultural Research (ISNAR), The Hague

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J-M (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16

John B, Yamashita T, Ludwig B, Flessa H (2005) Storage of organic carbon in aggregate and density fractions of silty soils under different types of land use. Geoderma 128:63–79

Johri BN (2001) Technology development and demonstration of a new bacterial inoculant (GRP3) for improved legume production. UP Govt., Project Report

Karlen LD, Buhler DD, Ellusbury MM, Andrews SS (2002) Soil, weeds and insect management strategies for sustainable agriculture. J Biol Sci 2:58–62

Kassam A, Friedrich T, Shaxson F, Pretty J (2009) The spread of conservation agriculture: justification, sustainability and uptake. Int J Agric Sustain 7:292–320

Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu Rev Microbiol (Ornston LN, Balows A, Gottesman-Susan E, eds), 211–236

Khan MA, Marwat KB, Hassan G, Shah WA (2002) Effect of different weed free periods on the growth and yield of wheat. Pak J Agric Agric Eng Vet Sci 18:30–33

Khan I, Hassan G, Khan MI, Khan IA (2004) Efficacy of some new herbicidal molecules on grassy and broadleaf weeds in wheat-II. Pak J Weed Sci Res 10:33–38

Kiers ET, West SA, Denison RF (2002) Mediating mutualisms: farm management practices and evolutionary changes in symbiont co-operation. J Appl Ecol 39:745–754

Kouno K, Wu J, Brookes PC (2002) Turnover of biomass C and P in soil following incorporation of glucose or ryegrass. Soil Biol Biochem 34:617–622

Lucy M, Reed E, Glick BR (2004) Application of free living plant-growth promoting rhizobacteria. Antonie van Leeuwenhoek Int J Gen Mol Microbiol 86:1–25

Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461

Marschner P, Yang CH, Lieberei R, Crowley DE (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biol Biochem 33:1437–1445

Morgan JAW, Bending GD, White PJ (2005) Biological costs and benefits to plant-microbe interactions in the rhizosphere. J Exp Bot 569:1729–1739

Nakamoto T, Yamagishi J, Miura F (2006) Effect of reduced tillage on weeds and soil organisms in winter wheat in summer maize cropping on humic andosols in central Japan. Soil Tillage Res 85:94–106

Oehl F, Sieverding E, Mader P, Dubois D, Ineichen K, Boller T, Wiemken A (2004) Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. Oecologia 138:574–583

Peacock AD, Muller MD, Ringelberg DB, Tyler DD, Hedrick DB, Gale PM, White DC (2001) Soil microbial community resources to dairy manure or ammonium nitrate applications. Soil Biol Biochem 33:1011–1019

Pregitzer KS, Zak DR, Loya WM, Karberg NJ, King JS, Burton AJ (2006) The contribution of root systems to biogeochemical cycles in a changing world. In: Cardon ZG, Whitebeck JL (eds) The rhizosphere: an ecological perspective. Elsevier, Oxford, pp 155–178

Richter DB, Oh NH, Fimmen R, Jackson J (2006) The rhizosphere and soil formation. In: Cardon ZG, Whitebeck JL (eds) The rhizosphere: an ecological perspective. Elsevier, Oxford, pp 179–200

Rillig MC (2004) Arbuscular mycorrhizae, glomalin, and soil aggregation. Can J Soil Sci 84:355–363

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis (DGGE): plant-dependent enrichment and seasonal shift revealed. Appl Environ Microbiol 67:4742–4751

Smeding FW, de Snoo GR (2003) A concept of food-web structure in organic arable farming systems. Landsc Urban Plan 65:219–236

Squartini A (2003) Functional ecology of the Rhizobium-legume symbiosis. In: Pinton R, Varanini Z, Nannipieri P (eds) The rhizosphere: biochemistry and organic substances at the soil plant interface. Marcel Dekker, New York, pp 297–326

Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old manure-treated agro-ecosystem. Appl Environ Microbiol 70:5868–5874

Tilak KVBR, Ranganayaji N, Pal KK, De R, Saxena AK, Nautiyal CS, Mittal S, Tripathi AK, Johri BN (2005) Diversity of plant growth and soil health supporting bacteria. Curr Sci 89:136–150

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418:671–677

Treseder KK, Turner KM (2007) Glomalin in ecosystems. Soil Sci Soc Am J 71:1257–1266

Vance CP, Unde SC, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resources. New Phytol 157:423–447

Vessey JK (2003) Plant growth-promoting rhizobacteria as biofertilizers. Plant and Soil 255:571–586

Whipps JM (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bot 82:1198–1227

Wright SF, Green VS, Cavigelli MA (2007) Glomalin in aggregate size classes from three different farming systems. Soil Tillage Res 94:546–549

Yang CH, Crowley DE (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl Environ Microbiol 66:345–351

Zhu Y-G, Miller RM (2003) Carbon cycling by arbuscular mycorrhizal fungi in soil– plant systems. Trends Plant Sci 8:407–409

Sustainable Enzyme Technology for Environment: Biosensors for Monitoring of Pollutants and Toxic Compounds

4

Pratyoosh Shukla, Vinod Nigam, Rishi Gupta, Ajay Singh, and Ramesh Chander Kuhad

Abstract

The recent developments in enzyme technology have raised the interest of researchers toward developing cost-effective enzyme-based techniques against various pollutants in our environment. Enzyme-based biosensors have diversity of industrial relevance in environmental monitoring. A variety of laboratory sample biosensors have been described recently which moderately measure variety of environmental pollutants. It is envisaged that many reports on biosensor development are directed toward their medical use, so the need of biosensor development for environmental applications and monitoring is growing. The present appraisal highlights recent major research findings on enzyme-based biosensors for determination of environmental pollutants and toxic chemicals.

Keywords

Enzyme technology • Biosensor • Pollutants • Environmental applications • Toxic compounds

Introduction

A biosensor is defined as an analytical device comprising of a biological catalyst or receptor in intimate contact with a suitable transducer such as an electrode and optical fiber (Brooks et al. 1991). The biological component is usually immobilized at or

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near to the surface of the transducer, which converts the particular biochemical event into a quantifiable and readily processable signal (Sethi 1994). According to IUPAC, it is a compact analytical device incorporating a biological or biologically derived sensing element either integrated within or intimately associated with a physicochemical transducer. The aim of a biosensor is to produce either discrete or continuous digital electronic signals, which are proportional to a single analyte.

Biosensor devices may have the capability to provide an analytically powerful and inexpensive alternative to conventional technologies by identifying the target analyte in the presence of interfering species. Reagent-less continuous real-time analysis is also one of the potent advantages offered by these devices. Microbial sensors are devices where microorganisms are used as a sensing element that can specifically recognize the species of interest either intimately connected or integrated within a suitable transducing system (Karube and Nakaniki 1994). Two major types of microbial sensors are being applied, one which involves the respiratory activity, while other uses the metabolites released by the microbes (Reidel et al. 1989). The best example of respiratory activity measurement is with DO probe and metabolite measurement with CO_2 electrode, fuel cell, NH_4 electrode, pH electrode, etc. The advantages of microbial sensors include less sensitivity to inhibition, tolerance to suboptimal pH and temperature of enzyme electrode, longer shelf life, and cheaper in manufacturing, while the disadvantages include specificity due to multienzyme system in cells and longer response time due to mass transfer resistance. Moreover, a noteworthy update about development of a new antibody-based small and sturdy "biosensor" developed to detect marine pollutants like oil much faster and more cheaply than current technologies is also quite impressive toward finding solutions to environmental problems (Spier et al. 2011)

Phenolic compounds are major pollutants in ground and surface water as they are widely used in many industrial processes such as plastic manufacture, resins, wood industry, construction industry, abrasives, plasticizers, cleaning products, pesticide manufacturing, and detergent industries (Lin et al. 2008; Gardziella et al. 2010; Apetrei et al. 2011). Taking into consideration their high toxicity and persistence in the environment, the determination of phenolic compounds becomes an important subject.

Since biosensor technology development demands rapid, inexpensive, and continuous monitoring capabilities, research endeavors to harmonize these issues are important. Biosensors currently developed or in the process of development are for detection of environmental pollutants, viz., phenols, genotoxins, and pesticides such as organophosphates and 2, 4-D.

This chapter highlights the advances in the rapidly developing area of microbial biosensors with particular emphasis to the developments since 2000 as a number of reports on biosensor development have been published during the last decade.

Classification of Biosensor

A biosensor is an analytical device for the detection of an analyte that combines a biological component with a physicochemical detector component with three parts:

(a) Sensitive biological material (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids)
(b) Biomimic or biomaterial
(c) Transducer as the detector that can be more easily measured and quantified

There are two main categories of biosensors described in literature (Amine et al. 2006).

Immobilization-Based Biosensors

In this method, the whole cells are used as the biochemical component (Rekha et al. 2000; Durrieu et al. 2004; Chouteau et al. 2005). This category of biosensor can increase the sensor steadiness and provide enzyme regeneration, where the sensing device is attached with matrix of immobilized enzyme (Lee et al. 2002). The lasting activity of the enzyme is estimated by quantifying the product. Nevertheless, these may have problems due to parallel reactions of several enzymes.

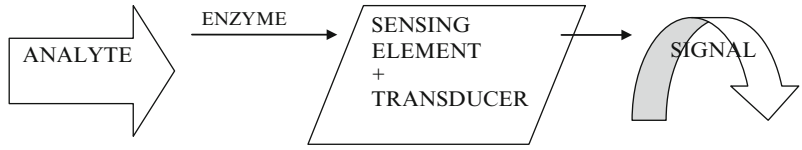

Fig. 4.1 A biosensor setup

Transducer-Based Biosensors

They are based on direct enzyme immobilization on a transducer device. The enzyme and transducer elements are in close contact with each other and incorporated in a single unit. Some biosensors based on enzyme inhibition have been reported in the literature (Tran-Minh 1985; Evtugyn et al. 1999; Luque de Castro and Herrera 2003).

In such biosensors, the enzyme (element) reacts with substrate, and biosensor response is assessed by the product concentration (P) of enzymatic reaction on sensor surface (Guilbault et al. 2004). The reaction is controlled by the rate of two synchronized processes, viz., enzymatic conversion of substrate (S) and diffusion of product. A simple setup is represented in Fig. 4.1 explaining the working principle of a biosensor.

Table 4.1 General requirements for environmental biosensors

Requirement	Specification range
Cost	$1–15 per analysis
Equipment portability	Can be carried by one person; no external power
Assay time	1–60 min
Personnel training	1–2-h training period is sufficient
Format	Reversible, continuous, in situ
Matrix	Minimal preparation for groundwater, soil extract, blood, and urine
Sensitivity	Parts per million (ppm) to parts per billion (ppb) concentration
Dynamic range	At least two orders of magnitude
Specificity	*Enzymes/receptors/nucleic acids:* specific to one or more groups of related compounds
	Antibodies: specific to one compound or closely related group of compounds

Adapted from Rogers and Gerlach (1996)

Parameters for Development of Biosensor

The stability and reproducibility of the biosensor are the most important parameters that depend on the response rate limiting step, substrate concentration, pH, strength of buffer, temperature, organic solvents, addition of additives and dry or wet storage, etc. Although some biosensors have been reported usable under laboratory conditions for periods of more than 1 year, their practical lifetime when incorporated into industrial processes or to biological tissue, such as glucose biosensors implanted in vivo, is either unknown or limited to days or weeks. It is necessary to emphasize that some precise conditions are required to be met for each environmental monitoring field (Table 4.1).

While it is relatively easy to determine the stability of biosensors at the laboratory scale, both during storage and operation in the presence of analyte, procedures for assessing their behavior during several days of introduction into industrial reactors are much more complex to handle. In both cases, it is advisable to specify the storage (shelf) or operational (use) lifetime and the storage and operating conditions in terms of buffer composition, presence of additives and substrate concentration (K_m), initial sensitivity, upper limit of concentration range for calibration, accuracy, and repeatability. The lifetime (LT) of a biosensor is defined as a comparative sensitiveness of different biosensors, developed from the same production batch of homogeneous patterns, after different storage conditions. Alternatively, biosensor stability is also measured as drift rate and useful for biosensors in which sensitivity evolution is either very slow or studied for short periods of time. The various advantages and disadvantageous of enzyme-based biosensor are summarized in Table 4.2.

Table 4.2 Advantages and disadvantages of enzyme-based biosensors

Advantages	Disadvantages
More specific than cell-based sensors	More expensive to produce due to the additional steps involved in isolating the enzyme
Faster response due to shorter diffusion paths (no cell walls)	Enzymes are often unstable when isolated
	Many enzymes need cofactors for their activity and detection of substances

Recent Trends in Biosensor Technology

Various novel developments in biosensor technology and their applications in the field of biotechnology are summarized in this section (Table 4.3). Abdelwahab et al. (2010) have described a nitric oxide nano-composite biosensor immobilizing microperoxidase (MP) toward determination of NO released from rat liver, stomach (AGS), and intestinal (HT-29) cancer cells. A notable work by Apetrei et al. (2011) was based on detection of phenolic compounds using amperometric tyrosinase-based biosensor. Further, for detection of organophosphate pesticides, Crew et al. (2011) developed an amperometric biosensor based on six acetylcholineste-rase enzymes through neural network program. In addition, there are prominent reports on development of tyrosinase-based biosensor for determination of o-diphenols (Daniela et al. 2010), air toxicity monitoring where bioluminescent bacteria were used as bioreporter through bioluminescence assay (Evgeni et al. 2011), algae-based biosensor for determination of environmental impurities of water (Dieter et al. 1998), and DNA biosensor for detection of mercury ions (Long et al. 2011). Recently application of nano-biotechnological tools has been reported for the evaluation of pollutants, e.g., development of a glucose biosensor using covalent cross-linking technique through carbon nanotubes hybrids for glucose estimation (Fu et al. 2011), and a nucleic acid biosensor (NAB) based on horseradish peroxidase (HRP) enzyme for diagnosis of genetic diseases and detection of infectious agents (He et al. 2011). Interestingly, Masojidek et al. (2011) have reported a novel method for measurement of herbicide toxicity and have successfully used this technique for detection of photosynthetic herbicides, e.g., diuron, atrazine, and isoproturon. In this series, other notable biosensors reported are the following: microbial biosensor for detection of methyl parathion (Kumar and D'Souza 2010), determination of hydrogen peroxide (Zhang et al. 2009; Li et al. 2009), amperometric polyphenol biosensor based on covalent immobilization of laccase onto copper nanoparticles for measurement of total polyphenolic content in plant extracts (Chawla et al. 2011), antibody-based KinExA Inline biosensor for estimation of dissolved polycyclic aromatic hydrocarbons (PAHs) (Spier et al. 2011), electrochemical detection of α-ketoglutarate (Poorahong et al. 2011), and amperometric fructose biosensor based upon the D-fructose dehydrogenase (FDH) toward determination of fructose in the real samples of fruit juice, soft drinks, and honey (Trivedi et al. 2009).

Moreover, few diversified remarkable studies like enzyme-amplified electrochemical biosensor through detection of PML–RARα fusion gene for diagnosis of promyelocytic leukemia (Lin et al. 2011), electrochemical DNA biosensor for screening of chlorinated benzene pollutants (Wu et al. 2011), enzyme biosensor based on chemiluminescence system, and using flowerlike ZnO crystals and nano-sized gold particles (Zhang et al. 2009; Yu et al. 2010) are prominent examples of recent knowledge revelation in biosensor development, making significant contribution for emergent biosensor industry for monitoring of pollutants and toxic compounds. Nevertheless, for efficient developments in sustainable enzyme technology, much of the efforts are needed for designing low-cost, ultrasensitive, selective, and quick-response biosensors. Considering above facts and with current advances in enzyme-based biosensor technology, we understand that such biosensors will have strong and momentous role in future development in biotechnology.

Table 4.3 A snapshot review of latest developments (2009–2011)

Type of biosensor technology	Working mechanism	Use	Reference
Nitric oxide nano-composite biosensor	Immobilizing microperoxidase (MP) onto the MWCNT-poly-carboxylic acid (PTTCA) nano-composite	Determination of NO released from rat liver, stomach (AGS), and intestinal (HT-29) cancer cells	Abdelwahab et al. (2010)
Amperometric tyrosinase	Electropolymerized phosphate-doped polypyrrole film as an immobilization support	Detection of phenolic compounds	Apetrei et al. (2011)
Amperometric biosensor	Array incorporated based on six acetylcholinesterase enzymes through neural network program	Determination of organophosphate pesticide	Crew et al. (2011)
Tyrosinase-based biosensor	Optical transparent support immobilized by "layer-by-layer" assembly, alternating the enzyme with the polycation polymer poly (dimethyldial-lylammonium chloride)	Determination of phenolics, e.g., o-diphenols	Daniela et al. (2010)
Algae-based biosensor	Optical immobilized living algae chlorophyll fluorescence depending on the load of water probes	Determination of environmental impurities of water	Dieter et al. (1998)
Fiber optics-based biosensor	Bioluminescent bacteria were used as bioreporter through bioluminescence assay	Air toxicity monitoring	Evgeni et al. (2011)
DNA biosensor	Evanescent wave all-fiber bisensing platform	Detection of mercury ions	Long et al. (2011)
Glucose biosensor	CS-incorporated sol–gel process, covalent cross-linking technique through carbon nanotubes hybrids	Glucose estimation	Fu et al. (2011)
Nucleic acid biosensor (NAB)	Based on horseradish peroxidase (HRP)–gold nanoparticle (Au-NP) dual labels and lateral flow strip biosensor (LFSB)	Detection of nucleic acid samples, diagnosis of genetic diseases, and detection of infectious agents	He et al. (2011)
Electrochemical PS II biosensor	Phytotoxicity index (PI) as a measure of herbicide toxicity	Detection of photosynthetic herbicides diuron, atrazine, and isoproturon	Masojidek et al. (2011)
Optical microbial biosensor	Using *Sphingomonas* sp. immobilized on microplate	Detection of methyl parathion	Kumar and D'Souza (2010)
Enzyme-amplified electrochemical biosensor	Through detection of PML–RARα fusion gene	Diagnosis of promyelocytic leukemia	Lin et al. (2011)
Amperometric enzyme biosensor	Based on in situ electrosynthesized gold/polyaniline core–shell nano-composites on conducting ITO electrode	Determination of hydrogen peroxide	Li et al. (2009)
Amperometric polyphenol biosensor	Based on covalent immobilization of laccase onto copper nanoparticles	Measurement of total polyphenolic content in plant extracts	Chawla et al. (2011)
Antibody-based biosensor	KinExA Inline sensor employed the monoclonal anti-PAH antibody	Assessments of dissolved polycyclic aromatic hydrocarbons (PAHs)	Spier et al. (2011)
α-ketoglutarate biosensor	Co-deposition of Ru and Rh nanoparticles over CFE	Real-time electrochemical detection of α-ketoglutarate	Poorahong et al. (2011)

(continued)

Table 4.3 (continued)

Type of biosensor technology	Working mechanism	Use	Reference
Amperometric fructose biosensor	Based upon the enzyme d-fructose dehydrogenase (FDH)	Determination of fructose in the real samples of fruit juice, soft drinks, and honey	Trivedi et al. (2009)
Electrochemical DNA biosensor	Based on double-stranded DNA modified Au electrode (dsDNA/Au)	Screening of chlorinated benzene pollutants	Wu et al. (2011)
Enzyme biosensor	Based on chemiluminescence system and hollow silica microspheres (HSM) receptor	Determination of glucose in human serum	Yu et al. (2010)
Enzyme-based biosensor	Using flowerlike ZnO crystals and nano-sized gold particles	Determination of hydrogen peroxide	Zhang et al. (2009)

References

Abdelwahab AA, Wei Choon Alvin Koh WCA, Noh HB, Yoon-Bo Shim YB (2010) A selective nitric oxide nanocomposite biosensor based on direct electron transfer of microperoxidase: removal of interferences by co-immobilized enzymes. Biosens Bioelectron 26:1080–1086

Amine A, Mohammadi H, Bourais I, Palleschi G (2006) Enzyme inhibition-based biosensors for food safety and environmental monitoring. Biosens Bioelectron 21:1405–1423

Apetrei C, Rodríguez-Méndez ML, De Saja JA (2011) Amperometric tyrosinase based biosensor using an electropolymerized phosphate-doped polypyrrole film as an immobilization support. Application for detection of phenolic compounds. Electrochim Acta 56:8919–8925

Brooks SL, Higgins IJ, Newman JD, Turner APF (1991) Biosensors for process control. Enzyme Microb Technol 13:946–955

Chawla S, Rawal R, Pundir CS (2011) Fabrication of polyphenol biosensor based on laccase immobilized on copper nanoparticles/chitosan/multiwalled carbon nanotubes/polyaniline-modified gold electrode. J Biotechnol 156:39–45

Chouteau C, Dzyadevych S, Durrieu C, Chovelon J-M (2005) A bienzymatic whole cell conductometric biosensor for heavy metal ions and pesticides detection in water samples. Biosens Bioelectron 21:273–281

Crew A, Lonsdale D, Byrd N, Pittson R, Hart JP (2011) A screen-printed, amperometric biosensor array incorporated into a novel automated system for the simultaneous determination of organophosphate pesticides. Biosens Bioelectron 26:2847–2851

Daniela F, Anna G, Daniela F, Gerardo P, Antonia M (2010) Mushroom tyrosinase in polyelectrolyte multilayers as an optical biosensor for o-diphenols. Biosens Bioelectron 25:2033–2037

Dieter F, Adrian M, Dieter B (1998) Detection of environmental pollutants using optical biosensor with immobilized algae cells. Sens Actuat B-Chem 51(1–3): 256–260

Durrieu C, Chouteau C, Barthet L, Chovelon J-M, Tran-Minh C (2004) A bi-enzymatic whole-cell algal biosensor for monitoring water pollutants. Anal Lett 37:1589–1599

Evgeni E, Vladislav P, Matan B, Robert SM (2011) Creation of a fiber optic based biosensor for air toxicity monitoring. Sens Actuat B-Chem 155:859–867

Evtugyn GA, Budnikov HC, Nikolskaya EB (1999) Biosensors for the determination of environmental inhibitors of enzymes. Russ Chem Rev 68:1041–1064

Fu G, Yue X, Dai Z (2011) Glucose biosensor based on covalent immobilization of enzyme in sol–gel composite film combined with Prussian blue/carbon nanotubes hybrid. Biosens Bioelectron 26:3973–3976

Gardziella A, Pilato LA, Knop A (2010) Phenolic resins: chemistry, applications, standardization, safety and ecology, 2nd edn. Springer, Berlin/Heidelberg

Guilbault GG, Pravda M, Kreuzer M (2004) Biosensors - 42 years and counting. Anal Lett 37:14481–14496

He Y, Zhang S, Zhang X, Baloda M, Gurung AS, Xu H, Zhang X, Liu G (2011) Ultrasensitive nucleic acid biosensor based on enzyme–gold nanoparticle dual label and lateral flow strip biosensor. Biosens Bioelectron 26:2018–2024

Karube I, Nakaniki K (1994) Immobilized cells used for detection and analysis. Curr Opin Biotechnol 5:54–59

Kumar J, D'Souza SF (2010) An optical microbial biosensor for detection of methyl parathion using *Sphingomonas* sp. immobilized on microplate as a reusable biocomponent. Biosens Bioelectron 26:1292–1296

Lee H-S, Kim Y, Chi Y, Lee TY (2002) Oxidation of organophosphorus pesticides for the sensitive detection by cholinesterase-based biosensor. Chemosphere 46:571–576

Li N-F, Lei T, Ouyang C, He Y-H, Liu Y (2009) An amperometric enzyme biosensor based on in situ electrosynthesized core–shell nanoparticles. Synth Met 159:1608–1611

Lin Y, Shi Y, Jiang M, Jin Y, Peng Y, Lu B, Dai K (2008) Removal of phenolic estrogen pollutants from different sources of water using molecularly imprinted polymeric microspheres. Environ Pollut 153:483–491

Lin L, Liu Q, Wang L, Liu A, Weng S, Lei Y, Chen W, Lin X, Chen Y (2011) Enzyme-amplified electrochemical biosensor for detection of PML–RARα fusion gene based on hairpin LNA probe. Biosens Bioelectron 28:277–283

Long F, Gao C, Shi HC, He M, Zhu AN, Klibanov AM, Gu AZ (2011) Reusable evanescent wave DNA biosensor for rapid, highly sensitive, and selective detection of mercury ions. Biosens Bioelectron 26: 4018–4023

Luque de Castro MD, Herrera MC (2003) Enzyme inhibition-based biosensors and biosensing systems: questionable analytical devices. Biosens Bioelectron 18:279–294

Masojidek J, Soucek P, Machova J, Frolik J, Klem K, Maly J (2011) Detection of photosynthetic herbicides: algal growth inhibition test vs. Electrochemical photosystem II biosensor. Ecotoxicol Environ Saf 74:117–122

Poorahong S, Santhosh P, Ramírez GV, Tseng T-F, Wong JI, Kanatharana P, Thavarungkul P, Wang J (2011) Development of amperometric α-ketoglutarate biosensor based on ruthenium–rhodium modified carbon fiber enzyme microelectrode. Biosens Bioelectron 26: 3670–3673

Reidel K, Renneberg R, Wollenberger U, Kaiser G, Scheller FW (1989) Microbial sensors: fundamentals and application for process control. J Chem Technol Biotechnol 44:85–106

Rekha K, Gouda MD, Thakur MS, Karanth NG (2000) Ascorbate oxidase based biosensor for organophosphorus pesticide monitoring. Biosens Bioelectron 15:499–502

Rogers KR, Gerlach CL (1996) Environmental biosensors: a status report. Environ Sci Technol 30:486A–491A

Sethi RS (1994) Transducer aspects of biosensors. Biosens Bioelectron 9:243–264

Spier CR, Vadas GG, Kaattari SL, Unger MA (2011) Near real-time, on-site, quantitative analysis of PAHs in the aqueous environment using an antibody-based biosensor. Environ Toxicol Chem 30:1557–1563

Tran-Minh C (1985) Immobilized enzyme probes for determining inhibitors. Ion Sel Electrode Rev 7:41–75

Trivedi UB, Lakshminarayana D, Kothari IL, Patel PB, Panchal CJ (2009) Amperometric fructose biosensor based on fructose dehydrogenase enzyme. Sens Actuator B 136:45–51

Wu L, Lu X, Jin J, Zhang H, Chen J (2011) Electrochemical DNA biosensor for screening of chlorinated benzene pollutants. Biosens Bioelectron 26: 4040–4045

Yu J, Ge L, Dai P, Ge S, Liu S (2010) A novel enzyme biosensor for glucose based on rhodanine derivative chemiluminescence system and mesoporous hollow silica microspheres receptor. Biosens Bioelectron 25(9):2065–2070

Zhang Y, Zhang Y, Wang H, Yan B, Shen G, Yu R (2009) An enzyme immobilization platform for biosensor designs of direct electrochemistry using flower-like ZnO crystals and nano-sized gold particles. J Electroanal Chem 627(1–2):9–14

Enzymatic Retting: A Revolution in the Handmade Papermaking from *Calotropis procera*

5

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Abstract

The rapidly changing economic and environmental needs of the society are putting ever-increasing pressure on the forest-based industry "To do more with less." This means increasing an efficient use of the available fiber resources producing more fiber on a shrinking land base using environmentally friendly processes and technologies and also more use of the nonwoody lignocellulosic fibers for industrial products. Continuously increasing demand and rising cost of traditionally used cellulosic raw materials by handmade paper industry like hosiery waste/cotton rags has forced the industry to look for alternate and locally available nonwoody lignocellulosic raw materials with the application of environmental-friendly processes. As a part of promotion of the green and clean technologies, Kumarappa National Handmade Paper Institute (KNHPI) focused its research and development activities in the area of identification of alternate lignocellulosic raw materials with integration of biotechnological approach, employing identified microorganisms and/or enzymes suitable for specific applications. *Calotropis procera*, locally known as ankra, which is available as a wild shrub in the desert area of Rajasthan and Gujarat, has proved one such potential lignocellulosic raw material, finding usefulness for making specialty handmade paper and products. Extensive research carried out at the institute indicated a good possibility of extraction of good quality bast fiber with the application of identified enzymes (bioretting process), making the process easier, productive, and less polluting while improving the yield and quality of the fiber obtained. The initiatives taken at KNHPI for utilization of *Calotropis procera* in the area

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of fiber extraction using bioretting process developed at the institute and utilization of the extracted fiber for making handmade paper and products should prove to be a potential employment-generating and income-generating opportunity among the rural masses. Proper utilization of the waste biomass, majority of which is presently utilized as a domestic fuel, could also improve the environmental status besides addressing the problem of global warming.

Keywords

Handmade paper • *Calotropis procera* • Lignocellulosic raw material • Plant fibers • Bast fibers • Retting • Enzymatic retting • Pectinases

Introduction

The Indian handmade paper industry has been identified as one of the village industry. And the industry has seen significant growth in the last one decade because of the improved demand not only on national level but also at the international arena. As per estimates, there are nearly more than 450 handmade paper units scattered all over India, producing nearly 50,000 tonnes of handmade paper and board. The Indian handmade paper industry had grown remarkably in the recent past, wherein the production of handmade paper industry has reached a turnover of US $ 50 million.

Due to increased literacy, industrialization, and modernization, the per capita consumption of the paper and paperboard has increased remarkably from 4.5 kg in the year 2000 to nearly 6.5 kg in the recent past. Around 10,000 persons are employed with the handmade paper industry. Most of them are located in rural areas. The remarkable point to be noted here is that the per capita consumption of paper and paper-board in India is low compared to the developed countries like USA (350 kg), China (58 kg), Japan (222 kg) and world average (55 kg) but the total quantity of paper produced in India is quite significant with a total production of nearly 7 million tonnes per annum.

Indian handmade paper industry contributes to less than 0.5% of the total country's production of paper and paperboard till the year 2005–2006, and this share has recently increased to nearly 0.7% in the recent year since the production of handmade paper which was nearly 25,000 tonnes in the year 2005–2006 and now stands at nearly 50,000 tonnes (Table 5.1). Although the country is self-sufficient in the manufacturing of handmade paper and board, however, a little quantity of specialty paper is imported.

Table 5.1 An overview of the Indian handmade paper industry

Particulars	2005–2006	2006–2007
Estimated production (tonnes)	25,000	50,000
Contribution in total P&B production %	0.4	0.6
Turnover, total P&B (Rs. Lakhs)	1,800,000	2,100,000
Turnover HMP (Rs. Lakhs)	12,500	20,000
Total export P&B (tonnes)	323,000	350,000
Exports HMP (tonnes)	20,906	42,000
Contribution of exports of HMP in P&B % (qty)	6.4	12
Value of exports total P&B (Rs. Lakhs)	124,639	147,500
Value of exports of HMP (Rs. Lakhs)	7,723	15,717
Contribution of exports of HMP in P&B % (value)	6.2	10.7
Imports HMP (tonnes)	3,755	2,800
Value of imports of HMP (Rs Lakhs)	847.7	518.6

Handmade paper production requires low capital investments. Thus, it is easy for small and local entrepreneurs to establish this industry in rural areas. This industry helps in generating employment at the local level. Since the handmade paper is tree-free, its manufacture does not result in the depletion of natural resources and causes much less pollution than factories making conventional paper. Handmade paper industry helps create sustainable livelihoods in rural areas. It employs unskilled workers, who are trained in these plants.

Thus, this enables the local population, especially women, to get employment in these production units. The industry produces numerous types of handmade paper including watermark, filter paper and drawing sheets.

Need of Alternative Lignocellulosic Raw Materials for Handmade Paper Industry

In view of the scarce availability and rising cost of the traditionally used cellulosic raw materials and to meet the immediate requirement of the Indian handmade paper industry, the institute has focused its R&D activities in the areas of identification of low-cost alternate lignocellulosic raw materials and promotion of cleaner production techniques to improve competitiveness of the Indian handmade paper industry. These efforts of the institute have also helped in the generation of employment among the rural masses. For sustainability of the handmade paper industry in the long run, the use of locally available lignocellulosic raw materials may be essential. Further, some of these raw materials have been found to impart special characteristics to the paper like strength, smoothness, which is an essential requirement of some of the varieties of handmade paper. The use of these raw materials may also result in increased production at a lower cost.

With a small investment, a number of handmade papermaking clusters could be developed based on locally available lignocellulosic raw materials, namely, jute, sunn hemp, ankra, bhimal, banana stem, thus solving problem of unemployment and meeting future demand of paper without affecting the environment. Agro-fibers like jute, straw, and banana are added to the primary fiber to create mottled handmade paper. Handmade paper has greater tensile, bursting, tearing, and double-fold strength when compared with conventional mill-made paper. It does not become brittle as it ages. The paper is available in a saga of rich varieties, designs, shapes, and colors.

Kumarappa National Handmade Paper Institute since its inception has been engaged in the research and development activities for the benefit of Indian handmade paper industry. After exhaustive research and development work at the institute, KNHPI has identified a number of locally available raw materials and evaluated their potential for handmade paper by developing suitable process technologies for producing varieties of specialty grades of handmade paper. Some of the important lignocellulosic raw materials studied are banana fiber, jute fiber, sisal fiber, silk mulberry, bhimal fiber, pineapple, etc. Suitability of handmade paper from these fibers has been evaluated on bench as well as pilot scale. Now it is time that these technologies developed by KNHPI are transferred to the handmade paper industry to create confidence among the entrepreneurs and to take full advantage of the technologies developed at KNHPI.

Use of these alternative lignocellulosic raw materials, which basically involve bast and leaf fibers, may include chemicals during pulping and bleaching processes for effective delignification required for producing quality handmade paper. In contrary to this, the traditionally used raw materials like cotton hosiery waste and cotton rags do not require any chemicals during pulping, thereby retaining ecofriendly status. In order to retain the ecofriendly credentials of the handmade paper industry, there is an urgent need to develop ecologically compatible processes for pulping and bleaching these alternative lignocellulosic raw materials. In pursuit of developing such techniques by KNHPI, biotechnology covering enzymatic prebleaching of pulp using xylanase and laccases, enzymatic retting, bio-refining and biopulping could be found to be one of the

potential options for its applications in handmade paper sector.

The present paper narrates about the process of bioretting in the specific context of handmade paper industry. Bioretting is highly significant in the present scenario because with the inclination of consumers towards handmade papers made out of natural fibers, there is a need of the consistent supply of the natural fibers in an economical way. Since the quality of handmade paper produced from the fiber extracted from the lignocellulosic raw materials is found to be superior in terms of physicochemical and strength properties, which could find applications in value-added products, the extraction of fiber from these plant materials is an important process in the present context.

Looking into the wide availability of ankra plant in the desert areas of Rajasthan, KNHPI evaluated it as an alternate source of raw material for handmade papermaking and found it to be having a very good potential. Therefore, further studies were started on the aspects of fiber extraction from the stem and twigs of ankra plant using enzymes. This paper presents all the details about the bioretting of ankra with the pulping characteristics of the extracted fiber for making handmade paper.

Plant Material and the Fibers

Plant Cell Wall Polysaccharides

Plant cell walls consist of three types of layers: middle lamella, primary cell wall, and secondary cell wall. The middle lamella is the first layer formed during cell division and can also be seen as the space between the cell walls and as the connecting region between adjacent cells, binding cells together. It is composed mainly of pectic compounds and protein. In later stages of wood cell growth, middle lamella becomes highly lignified. This lignin works as an efficient and resistant glue to keep different cells together, while in many herbs and in the nonwoody tissues, pectin performs this function. The primary cell wall, a thin layer, is formed after the middle lamella and consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicelluloses, and glycoprotein.

Plant cell walls consist of a matrix of cellulose microfibrils, in most cases cross-linked by hemicellulose, pectin, and extension. Wood cells also contain lignin, a non-polysaccharide polymer. The composition of the noncellulose polysaccharides varies highly between different plants, cell types, and even cell wall layers. Polysaccharides in plant cell walls are the most abundant organic compounds found in nature and are the most important renewable natural resources. They constitute up to 90% of the plant cell wall and can be divided into three groups – cellulose, hemicellulose, and pectin.

In addition to cellulose and hemicellulose, pectin is an important component of dicotyledonous plant cell walls. Pectins belong to a class of complex, heterogeneous, and multifunctional polysaccharides, forming a hydrated cross-linked three-dimensional network in the matrix of primary plant cell walls, and play diverse roles in cell physiology, growth, adhesion, and separation. This interlinked network of pectin and hemicellulose helps adjacent cells to bind each other and cushion them. The pectin-rich region of the plant cell wall, the middle lamella, acts as a glue to hold adjacent cells together.

Calcium Ions

The calcium ions mainly function as constituents of the cell wall, the middle lamella of which consists largely of calcium pectate. The interactions between calcium ions and cell walls play a key role in plant physiology. Calcium ions are involved in many mechanisms, for example, stabilization of cell wall structures by cross-linking pectin chains, ion exchange properties, and control of the activities of wall enzymes. Grant and coworkers (1973) have proposed an eggbox type of model for calcium coordination in middle lamella, in which two helical PolyGalA fragments interact with each other via ionic interactions and coordination of calcium ions between chains. Calcium ion binding of pectic components has been found

to be a cooperative mechanism as the initial binding of a few calcium ions is responsible for the chains falling into lines, thus facilitating further calcium ion fixation. Thus, calcium ions connect two acidic polysaccharides, that is, cross-linking. Thus the so-called "calcium egg boxes" cross-linking smooth regions in pectin chains play an important role for keeping the middle lamella together in many non-lignified plant tissues. It has been reported that removal of these calcium ions may increase the susceptibility of pectin to enzymatic attack (Rihouey et al. 1995). Calcium chelators such as EDTA and Oxalic Acid have reported to be used successfully by Henriksson et al. (1997).

Plant Fibers

Plant fibers are long, narrow, thick-walled, and lignified sclerenchymatous cells, which are dead and therefore serve the purely mechanical function of giving strength and rigidity to the plant body. Plant fibers are classified into three main types according to their origin and structure: surface fiber, which is produced on the surface of stems, leaves, etc., for example, cotton (*Gossypium* sp.); hard or structural fibers, which are supportive and conductive fibrovascular bundles, chiefly found in monocots, for example, Manila hemp (*Musa textilis*); and soft or bast fibers, formed in groups outside xylem in the cortex, phloem, or pericycle, for example, Ramie, jute, paper mulberry, and sunn hemp.

Bast Fibers

Bast fiber or skin fiber is the fiber collected from the phloem (the "inner bark" or the skin) or bast surrounding the stem of certain, mainly dicotyledonic, plants. Most of the technically important bast fibers are obtained from herbs cultivated in agriculture, as, for instance, flax, hemp, or ramie, but also bast fibers from wild plants, as stinging nettle and trees as the lime tree, have been used to some extent. Since the valuable fibers are located in the phloem, they must often be separated from the xylem material (woody core) and sometimes also from epidermis. The process for this is called retting and can be performed by microorganisms either on land or in water (Allen 1946), or by chemicals (for instance high pH and chelating agents), or by pectinolytic enzymes. In the phloem bast fibers occur in bundles that are glued together by pectin and calcium ions. More intense retting separates the fiber bundles into elementary fibers that can be several cm long. The bast fibers have often higher tensile strength than other kinds and are therefore used for textiles, ropes, yarn, paper, composites, and burlap.

In retting, bast fiber bundles are separated from the core, the epidermis, and the cuticle. This is accomplished by the cleavage of pectins and hemicellulose in the flax cell wall, a process mainly carried out by the plant pathogens like filamentous fungi. The remaining bast fibers are mainly composed of cellulose and lignin. The transgenic flax plants have been generated with increased polygalacturonase and rhamnogalacturonase activities through constitutive expression of *Aspergillus aculeatus* genes in tissue-cultured and field-grown plants (Musialak et al. 2008). This resulted into a reduction in the pectin content, thereby showing a significantly higher retting efficiency of the transgenic plant fibers without affecting the fiber composition.

Extraction of Fibers

Conventional Process of Fiber Extraction

The process of extracting fiber from nonfibrous tissue is called retting. Retting is a microbial process that breaks the chemical bonds holding the stem together and allows separation of the bast fibers from the woody core. The process of retting is being utilized for centuries in traditional ways for extracting the fiber. There are mainly four kinds of retting processes which are (Van Sumere 1992):

1. Dew or field retting
2. Pool retting
3. Stream retting
4. Water retting

Dew or Field Retting

This is the oldest technique as retting of green and dried flax was used by the Egyptians thousands of years ago. Under this technique, plant stems are cut or pulled up and left in the field to rot by exposure to dew, rain, wind, and sunlight. The process is to be monitored closely to ensure that the bast fibers separate from the inner core without much deterioration in quality. Moisture is needed for the microbial breakdown to occur but then the weather must be dry for bailing. It is an inexpensive, mechanized procedure and does not use water. But it causes large variations in fiber quality; coarser and lower quality of fiber than with water retting, it has limitation to geographical regions with appropriate temperature and moisture, high labor costs, and occupation of agricultural land for several weeks.

Pool Retting (Blue Ret/Green Ret)

This method was also used by ancient Egyptians and described by Pliny in 79 A.D. It is one of the most rapid natural retting procedures. According to this method, flax, tied up as beets, was used to be lowered into the pool, weighed down with sods to keep it under water, and the microorganisms retted the flax within 1–3 weeks. This method was in use until 1920 and produced fibers with a grayish-blue appearance. So, it is also known as blue ret or green ret.

Stream Retting (Analogous To Pool Retting)

In this type of retting, flax was used to be submerged in slow running water, which does not contain as many bacteria as stagnant water. The method was used in the Netherlands and Germany. Due to the negative side effects, it was abandoned in 1937–1943. Despite the enormous economic benefits, it has disadvantages of river pollution, a sour, unpleasant smell, and navigational problems.

Water Retting

In principle, the method is analogous to stream retting, but the stems are retted for 4–7 days depending on the quality of the plant used in heated tanks or pits maintained at 40°C. Warm water retting, which can be used all year round, results normally in finer fibers of better quality than those produced by dew retting. Although it produces more uniform and high-quality fiber, the stench from fermentation by anaerobic bacteria and the resulting stench-tainted fibers and high labor costs prevent it from being used widely today. It has been largely abandoned in countries where strict environmental regulations exist because of the environmentally unacceptable fermentation waste.

Thus, the traditional retting process is quite tedious which involves treatment of the woody raw material under water in submerged conditions over a long period of 15–30 days and which also results in inferior quality fiber and creates problem of water pollution besides concern for global warming. So, an alternative method of fiber extraction is the need of the hour.

The bast fibers like ramie thus obtained are good natural textile material or papermaking raw materials but decorticated ramie or other such fibers contain 20–30% ramie gum, which consists mainly of pectic and hemicelluloses, and degumming is necessary to meet textile or other industrial requirements (Kashyap et al. 2001). Therefore, prior to industrial utilization of the processed bast fibers, removal of heavily coated, noncellulosic gummy material from the cellulosic part of fibers, that is, “degumming” is necessary. In a classical degumming process, the heavy coating of gums, waxes and pectin that remains on the processed bast fibers is removed. On an industrial scale, the degumming of bast fibers is carried out by treating the crude fibers with dilute lye solutions (12–20% NaOH and 2% NaOH in case of ramie and sunn hemp, respectively) containing wetting and reducing agents. Following a 24-h soaking period, the fibers are boiled for 1–4 h, rinsed, neutralized, washed, and centrifuged several times. The fibers are then dried over charcoal fire and treated with softeners such as gylcerine, wax, and soap to prevent the fibers from becoming brittle. The cleaned fibers are further graded and processed.

The chemical treatment of fibers is believed to produce pollution, toxin, and nonbiodegradable effluents and causes serious environmental threats and biological disturbances, not to mention the high consumption of energy. With rising energy costs due to scarcity of energy resources and deterioration of the environment beyond a toler-

able level, there is an urgent need to develop alternate, environmentally friendly processes for degumming and processing of bast fibers. Kapoor et al. (2001) have reported a comparative study of conventional chemical degumming process and enzymatic process of degumming for the bast fibers of ramie and sunn hemp.

Enzymatic Retting Process for Extraction of Fibers

To overcome the handicaps of dew and water retting, a new method of enzymatic retting or bioretting has been developed. It produces a better quality of fiber in higher yield and consistent quality. Moreover, it is faster than traditional retting, readily controlled, and produces fewer odors. But detailed study is required to make it competitive with the traditional methods.

Bioretting process, which involves the use of pectinase-based enzymes, holds greater promise for extraction of fiber. In the process of bioretting or enzymatic retting, the microbial enzymes produced by the retting agents during dew retting or in warm water retting are replaced by the industrially obtained enzymes mainly pectinases. Polygalacturonase plays a key role in the enzymatic retting of flax because a strong correlation has been found between the ability to perform retting and degradation of sparsely esterified pectin, a substrate of polygalacturonase. An extracellular polygalacturonase produced by *Rhizopus oryzae*, a very potent retting organism, has been purified and characterized. The enzyme is reported to be the single active component in retting and shown to have non-methylated polygalacturonan as its preferred substrate. It was further reported that the purified polygalacturonase (EC 3.2.1.15) belongs to glycosyl hydrolase family 28 to which most of the known polygalacturonases belong. However, it was reported to carry an N-terminal sequence that does not occur in other fungal pectinases, but is present in a polygalacturonase from the phytopathogenic bacterium, Ralstonia solanacearum, a parasite that causes severe damage (brown rot) to several plants including potato and ginger. (Zhang 2006; Henrissat 1991).

Pectic substances are ubiquitous in the plant kingdom and form the major components of middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells. The enzymes hydrolyzing these pectic substances are broadly known as pectinases and include polygalacturonases, pectin esterases, pectin lyases and pectate lyases depending on their mode of action. Pectinolysis is one of the most important processes for plants as it plays a role in cell elongation and growth as well as in fruit ripening. Pectolytic enzymes are widespread in nature and are produced by bacteria, fungi, yeasts, insects, nematodes and protozoa. Microbial pectolysis is important in plant pathogenesis, symbiosis, and plant deposits. Thus, by breaking down pectin polymer for nutritional purposes, microbial pectolytic enzymes play a hugely important role in nature (Hoondal et al. 2002). Pectinases can modify and degrade pectins, a class of heterogeneous and multifunctional polysaccharides present in middle lamella and primary cell walls of plants. Pectins have been shown to play diverse roles in cell physiology, growth, adhesion, and separation. Pectinases are used technically in the processing of fiber production and fruit juice or wine making.

Pectinases

Due to the complex structure of pectin, the pectin degrading enzymes, pectinases are actually a diverse group of enzymes that can modify and degrade pectin. Pectinases are classified into various groups as given below:

(a) According to their site of cleavage, pectinases are classified as endo or exo (if they cleave within or at the end of the substrate chain, respectively) on the basis of the mode by which they cleave the glycosidic bond; they are classified as hydrolase or lyase and on the basis of the substrate (pectin or pectate).

(b) According to the cleavage site, pectinases can be further divided into two groups, those acting on the main chain (endo- and exopolygalacturonase, pectin and pectate lyases, pectin and pectate lyases, rhamnogalacturonan hydrolases and lyases, pectin methyl and acetyl esterases, and rhamnogalacturonan

acetyl esterase) and those acting on side chains of the pectin hairy regions (arabinofuranosidases, endoarabinanase, β-galactosidase, endogalactanases, and feruloyl esterases).

(c) Depending on which part of the pectin chain is attacked, pectinases are classified as rhamnogalacturonase for the rhamnogalacturonic segment or polygalacturonase for the smooth region. The latter enzyme might be specialized on methylated or non-methylated regions, and it is probably the most studied pectinase and is produced by many organisms.

Role of Pectinases in Bioretting Process

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. Alkaline pectinases are among the most important industrial enzymes and are of great significance in the current biotechnological arena with wide ranging applications in textile processing, degumming of plant bast fibers, treatment of pectic wastewaters, papermaking, and coffee and tea fermentations.

The most upcoming application of pectinolytic enzymes is their use in the degumming and extraction of plant fibers such as ramie, sunn hemp, jute, flax, and hemp. Polysaccharide-degrading microorganisms and enzymes such as pectinases or a combination of pectinases and xylanases could be used to remove the gummy material from plant fibers. The processing of fiber is fast and degumming is much more specific.

Pectins, which are chemically complex-binding substances in plants, hold fibers in bundles and the bundles between nonfibrous tissues in the outer region of the stems. While most enzymes for retting purpose are mixtures of polysaccharidases, pectinases are essential for effective retting and constitute a large proportion of successful retting formulation. Pectinases are believed to play a leading role in the processing of fibers, since 40% of dry weight of plant cambium cells is comprised of pectin. Pectinases effectively assist in degumming, maceration, and retting of jute, flax, hemp, and ramie bast fibers by degrading the pectin located in the middle lamella and primary cell wall.

Among these enzymes are hydrolases and lyases, represented, respectively, by polygalacturonase (EC 3.2.1.15) and pectate lyase (EC 4.2.2.2.). Retting by hydrolases in particular has been reported and is still applied in industrial processes. The retting by the hydrolases investigated to date proceeds normally either in neutral or acidic conditions. Alkaline conditions, on the other hand, allow the plant tissues to swell, and the alkali brings about degradation of pectic substances by a non-enzymatic β-eliminative splitting reaction. Such modifications of tissue structures would be expected to enhance the substrate accessibility for pectolytic enzymes active at high pH. Pectate lyase (PAL) excreted by *Erwinia* species has an optimum pH for activity in the range of 8.0–9.5 and was shown to be of potential use for enzymatic pulping (Horikoshi 1990; Yoshihara and Kobayashi 1982).

Commercial cellulases, hemicellulases, pectinases, and other polysaccharidases have been applied to flax at various levels and compared to traditional retting methods (Sharma 1987). Pectinolytic enzymes secreted by soft-rot bacteria also cause maceration of woody fabrics that are long, strong, and usually stiff. Pectinolytic and xylanolytic enzymes help in softening of these fibers. The combined alkali and enzyme treatment improves fiber quality (Bajpai 1999; Beg et al. 2001; Kirk and Jeffries 1996; Hoondal et al. 2002).

There are several essential factors determining the efficacy of a potential pectinase producer to be used for degumming of plant fibers. In addition to the complete removal of pectic substances by the pectinase preparation from the potential degumming enzyme-producing strains, there should not be any reduction in the tenacity and strength of plant fibers during enzymatic retting, that is, strain should be cellulase negative. The pectinase enzyme should be active at neutral or in alkaline pH range (7–10).

Measurement of Retting Efficiency

The Fried test method which is basically used to determine the retting degree, is empirical (Dujardin 1942) and rather subjective in the hands of inexperienced operators. Therefore, a modified version

of the Fried test has been suggested to monitor the fiber separation. This modified procedure partly diminishes the subjective nature of the Fried test by introducing a "blind test" with an "independent" examiner and a relatively "objective" measurement.

According to this method, retting efficiency can be measured on a scale from 0–6 as explained by Zhang 2006. Therefore, the samples are visually graded on a scale from 0–6 on the basis of the degree of fiber release and separation from the stem twigs.

"0" The value of "0" indicates negligible retting implying that fibers can't be released at all.

"1" The value of "1" implies that bast fibers can be separated from 0–10 mm of the 10 cm long twigs.

"2" shows that fibers are released from 10–25 mm.

"3" shows fiber release from 25–50 mm.

"4" shows fiber release from 50–75 mm.

"5" implies that more than 75 mm of the fiber is released, but still connected via at least one point.

"6" means that all fibers are released totally.

To avoid bias, all samples are given random numbers and the grading is done by an examiner without knowledge of the exact treatment of the individual sample.

Extraction of Ankra Fiber

The enzymatic retting efficiency of the available pectinase enzyme sample (Meshzyme) was studied using the green stem of ankra plant, and a comparison was made with the traditional process of water retting. Besides, the effect of recycling of the retting liquor was also studied.

The pretreatment methods like acidic pretreatment or the boiling of ankra twigs was also studied to see their effect on retting efficiency of the pectinase enzyme in comparison with the water retting of the dried twigs of ankra.

The ankra fiber was extracted on bench scale utilizing the treatment conditions optimized with the dried twigs of ankra plant with sample pretreatment.

The ankra fiber obtained from bench-scale operation of the enzymatic retting process was used to study its pulping characteristics under different conditions. For this, a comparison of the pulp quality was made with the pulp obtained under similar conditions from the commercially available fiber of ankra.

The banana pulp obtained from APP process with 8% NaOH and 2% H_2O_2 was used to see the effect of its blending n the ankra pulp.

The green ankra twigs were used to evaluate the fiber extraction possibilities using the enzymatic retting process and its comparison to the conventional process of water retting. It was observed that it takes a period of 10–12 days to extract the fiber from ankra twigs immersed in water at ambient temperature. While a period of 1 day only was sufficient in the enzymatic route when the fresh retting liquor was used for the first time. But during the recycling of the same retting liquor for the second time, it took 2 days, and during recycling for the third time, it took 3 days for the similar softening of the ankra twigs. The fiber yield was found to be in the similar range for the enzymatically and nonenzymatically extracted fiber. But the point to be emphasized here is that the higher yield in the third case was found due to the direct wet harvesting of the fiber. So, it was found that the recycling is possible without any loss in fiber yield and that higher yield could be obtained when fiber is harvested immediately under wet conditions (Table 5.2).

While evaluating the fiber extraction possibilities from dried stem of ankra, it was found that slightly lower yields could be obtained both through the enzymatic and nonenzymatic route as compared to the green ankra twigs. Besides, the pretreatment of ankra twigs either through acid to a pH of 3–4 or through boiling for about an hour proved to be beneficial because it resulted into a better process of the enzymatic retting both in terms of fiber yield and the ease to extract fiber from the twigs (Table 5.3). It was further observed that the addition of EDTA could be omitted by the acidic pretreatment step at a pH of 3–4.

With the optimized conditions as given in Table 5.4, ankra fiber was extracted at the bench scale using the dried twigs after a slight hammering action. A fiber yield of 15% could be obtained. A slight lower yield was because among the dried

Table 5.2 Ankra fiber extracted from green stem through water and enzymatic retting

		Enzymatic retting		
Particulars	Water retting	Fresh retting liquor	Retting liquor recycled II time	Recycled liquor III time
Incubation time	12 days	1 day	2 days	3 days
Fiber yield	26%	27.8% (green fiber harvested after rewetting)	26.97% (fiber harvested after drying)	35% (fiber harvested wet directly)
Total solids in retting liquor	0.54%	0.4%	0.77%	1.82%

Table 5.3 Ankra fiber extracted from dried stem through enzymatic retting process

	A	B	C	D
Particulars	Enzymatic retting with EDTA (%)	Enzymatic retting with pH adjustment and without EDTA (%)	Boiling then enzymatic retting with EDTA (%)	Boiling then enzymatic retting with pH adjustment & without EDTA (%)
Fiber yield	21	25	18.4	20
Total solids	1.1	1.18	1.07	0.94

Table 5.4 Optimized treatment conditions for fiber extraction

Parameters	Values
Acidic pretreatment	pH of 3–4 for a period 2–3 h
Surfactant	0.1%
Enzyme (Meshzyme)	0.5%
pH after enzyme addition	5–6
Incubation period at ambient temp. (i.e., 25°C)	2–3 days

Table 5.5 Pulp yield and black liquor of ankra pulp from open digestion and APP

	Ankra pulp from open digestion -6% NaOH		Ankra pulp from APP- 5+3%		Ankra pulp from APP-6+3%	
Particulars	Commercial fiber (%)	Enzyme-retted fiber (%)	Commercial fiber (%)	Enzyme-retted fiber (%)	Commercial Fiber (%)	Enzyme-retted fiber (%)
Pulp yield	79	81.5	73	75	76	78
Total solids in black liquor	2.57	1.54	4.81	3.2	4.61	3.6

twigs used, there were much of the highly matured twigs which could give less fiber. So, it was observed that the twigs of medium age are best for extracting good amount of fiber.

The ankra fiber thus extracted enzymatically on bench scale was used further to study its pulping characteristics in comparison to that obtained from the commercial fiber. Both the open hot digestion and alkaline pulping process (APP) was used to pulp both the types of fibers. Pulp yield and black liquor properties of the pulps obtained are given in Table 5.5. The physical strength properties of the pulps thus obtained are listed in Table 5.6.

The pulp obtained from APP of the enzymatically extracted ankra fiber was used to see the influence of blending of banana pulp (APP). It

Table 5.6 Strength properties of ankra pulps from enzyme-retted and commercial fibers

	Enzyme-retted fiber		Commercial fiber	
Parameters	Open digestion	APP	Open digestion	APP
GSM	64	66	63	55
Burst index	2.083	2.27	3.03	3.33
Tear index	13.54	13.73	17.8	15.66
Tensile index	30.86	25.28	38.70	17.0
Double fold	168	204	1,314	2,567

Table 5.7 Blending studies of the enzyme-retted fiber of ankra

Parameters	Screened APP pulp	Screened APP with 20% banana	Pulp of open digestion with 20% banana pulp	Banana pulp used in blending
GSM	63	58	50	53
Burst index	1.32	1.49	2.0	3.14
Tear index	11.42	11.26	16.80	29.9
Tensile index	13.8	30.34	40.80	14.98
Double fold	100	1,105	282	3,800

was observed that the ankra pulp has the qualities equivalent to that obtained from banana fiber and its properties could be improved further by blending banana pulp (Table 5.7).

Conclusion

The process of enzymatic retting has proved to be a very useful process with the following benefits:

- A slightly higher yield of fiber through enzymatic route
- Reduction in the problem of water pollution
- Reduction in the time required to extract fiber conventionally
- Possibility to use the same enzyme/retting liquor twice or thrice for extracting the fiber from fresh lots of plant parts used
- A good quality handmade paper

The process of bioretting thus developed might help in providing a sustainable and consistent supply of natural fibers to the handmade paper industry besides creating a good employment potential in the rural areas of the country.

References

Allen LA (1946) Recent developments in the microbiology of retting Part I. The microbial flora in different systems of retting. J Soc Chem Ind 65:365–370

Bajpai P (1999) Applications of enzymes in the pulp and paper industry. Biotechnol Prog 15:147–157

Beg QK, Kapoor M, Tewari RP, Hoondal GS (2001) Bleach-boosting of eucalyptus kraft pulp using combination of xylanase and pectinase from Streptomyces sp QG-11-3. Res Bull Punjab Univ Sci 51:71–78

Dujardin A (1942) Vlas roten. De Westvlaamsche Boekhandel S.V. Groeninghe drukkerije, Kartrijk, Belgium

Grant GT, Morris ER, Rees DA, Smith PJC, Thom D (1973) Biological interaction between polysaccharides and divalent cations: the egg box model. FEBS Lett 32:195–198

Henriksson G, Akin DE, Henlin RT, Rodriguez C, Archibald DD, Rigsby LL, Eriksson KEL (1997) Identification and retting efficiencies of fungi isolated from dew-retted flax in the United States and Europe. Appl Environ Microbiol 63(10):3950–3956

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Hoondal GS, Tewari RP, Tewari R, Dhaiya N, Beg QK (2002) Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol 59:409–418

Horikoshi K (1990) Enzymes of alkalophiles. In: Fogarty WM, Kelly CT (eds) Microbial enzymes and biotechnology, 2nd edn. Elsevier Applied science, London, pp 275–294

Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001) Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotolaria juncea*) bast fibers. Process Biochem 36:803–807

Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector-a review. Bioresour Technol 77:215–227

Kirk TK, Jeffries TW (1996) Role of microbial enzymes in pulp and paper processing. In: Jeffries TW, Viikari L (eds) Enzymes for pulp and paper processing, ACS Symposium series. American Chemical Society, Washington, pp 1–14

Musialak M, Kwaitowska MW, Kulma A, Starzycka E, Szopa J (2008) Improving retting of fiber through genetic modification of flax to express pectinases. Transgenic Res 17(1):133–147

Rihouey C, Jauneau A, Cabin Flaman A, Demarty M, Lefebvre F, Morvan C (1995) Calcium and acidic pectin distribution in flax cell walls: evidence for different kinds of linkages in the cell wall junction and middle lamella of the cortical parenchyma of flax hypocotyl. Plant Physiol Biochem 33:497–508

Rognes H, Gellerstedt G, Henriksson G (2000) Optimization of flax fiber separation by leaching. Cellul Chem Technol 34:331–340

Sharma HSS (1987) Enzymatic degradation of residual non-cellulosic polysaccharides present on dew retted flax fibers. Appl Microbiol Biotechnol 26:358–362

Van Sumere CF (1992) Retting of flax with special reference to enzyme retting. In: Sharma HSS, Van Sumere CF (eds) The biology and processing of flax. M publications, Belfast, pp 157–198

Yoshihara K, Kobayashi Y (1982) Retting of mitsumata bast by alkalophillic bacillus in papermaking. Agric Biol Chem 46(1):109–117

Zhang J (2006) Biochemical study and technical applications of fungal pectinase. In: The Dean of faculty of Science and Technology (ed) Digital comprehensive summaries of Uppasala dissertations from the faculty of science and technology 137, Sweden. ISSN 1651–6214, ISBN 91-554-6441-6

Cellulases and Their Biotechnological Applications

6

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Abstract

For a long-range solution to the global issues of energy, chemical and food, the most abundant, renewable and sustainable bioresource cellulose could be a feasible solution. The depolymerisation of cellulose by a group of enzyme cellulases could potentially lead to the development of various value-added products. Due to their immense potential, cellulases are involved in various industrial and biotechnological applications related to pulp and paper, textile, fuel and other organic chemical synthesis industries. However, to further economise the cellulase production, extensive research is being carried out using various approaches including genetic manipulation and process engineering. In this chapter, a brief overview of cellulases and their potential applications are being discussed.

Keywords

Cellulase • Cellulose • Biotechnology • Pulp and paper industry • Textile industry • Fuel industry

Introduction

Cellulose is one of the most abundant, renewable and sustainable source of feedstock, which can be utilised for the development of various value-added products (Kuhad and Singh 1993, 2007; Kung et al. 1997; Kuhad et al. 2011; Gao et al. 2008). The annual production of cellulose has been estimated to be approximately 15×10^{12} t per year of the total biomass produced through photosynthesis. Structurally, cellulose is a fibrous, insoluble and a major crystalline polysaccharide constituent of plant cell walls, composed of repeating cellobiose units linked by β-1,4-glucosidic bonds (Jagtap and Rao 2005).

In nature, cellulose is used as a food source by a wide variety of organisms including fungi, bacteria, plants and protists as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, mollusks and nematodes (Watanabe and Tokuda 2001; Davison and Blaxter 2005).

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The complete cellulase system includes exo-β-1, 4-glucanases (EC 3.2.1.91), endo-β-1,4-glucanases (EC3.2.1.4) and β-1,4-glucosidase (EC 3.2.1.21) (Wilson and Irwin 1999). These enzymatic components act sequentially in a synergistic system to facilitate the breakdown of cellulose and the subsequent biological conversion to a utilisable energy source, glucose (Beguin and Aubert 1994).

Due to their immense potential, cellulases have been used in various industrial and technological applications. These include use of cellulases in deinking of paper waste, paper industry, textile industry, biopolishing and biostoning of denim jeans, food and feed industry, sugar and oligosaccharides production, biofuel production and production of other value-added commodities.

In recent years, fundamental and applied researches on cellulase enzyme have not only generated significant scientific knowledge but also have revealed their enormous potential in biotechnology, making significant advances towards the production and alteration technology of cellulase enzyme using several biotechnological approaches. A brief overview about cellulases, cellulolytic microbial strain improvement and their various biotechnological applications has been provided here.

Structure of Cellulose

Cellulose is a glucan polymer of D-glucopyranose units, which are linked together by β-1,4-glucosidic bonds. The cellulose has an average degree of polymerisation (DP) of at least 9,000–10,000 and possibly as high as 15,000. An average DP of 10,000 would correspond to a linear chain length of approximately 5 μm in wood. An approximate molecular weight for cellulose ranges from about 10,000 to 150,000 Da. Anhydrocellobiose is the repeating unit of cellulose. Coupling of adjacent cellulose chains and sheets of cellulose by hydrogen bonds and van der Waals forces results in a parallel alignment and a crystalline structure with straight, stable supramolecular fibres of great tensile strength and low accessibility (Demain et al. 2005; Nishiyama et al. 2003; Notley et al. 2004; Zhang and Lynd 2004). The cellulose molecule is very stable, with a half-life of 5–8 million years for β-glucosidic bond cleavage at 25°C. There are several types of cellulose in wood: crystalline and noncrystalline and accessible and non-accessible. Most wood-derived cellulose is highly crystalline and may contain as much as 65% crystalline regions. The remaining portion has a lower packing density and is referred to as amorphous cellulose. Accessible and non-accessible refer to the availability of the cellulose to water, microorganisms, etc. The surfaces of crystalline cellulose are accessible but the rest of the crystalline cellulose is non-accessible, whereas most of the noncrystalline cellulose is accessible but part of the noncrystalline cellulose is so covered with both hemicelluloses and lignin that it becomes non-accessible. Concepts of accessible and non-accessible cellulose are very important in moisture sorption, pulping, chemical modification, extractions and interactions with microorganisms.

Cellulases and Their Mechanism

Cellulases are generally defined as enzymes which hydrolyse the β-1, 4 glycosidic bonds within the chain that comprise the cellulose polymer. Fungal and bacterial cellulases significantly differ in their structure and functions. Fungal cellulases are composed of a carbohydrate-binding module (CBM) at the C-terminal joined by a short polylinker region to the catalytic domain at the N-terminal. The CBM is comprised of approximately 35 amino acid residues, and the linker region is a highly glycosylated region unusually rich in serine, threonine and proline amino acid residues (Divine et al. 1988). This linker region is also the site of proteolytic cleavage accomplished by several general serine proteases. Broadly, there are three types of cellulases:

1. Endoglucanase or carboxymethyl cellulase (E.C. 3.2.1.4)
2. Exoglucanase or cellobiohydrolase or filter paper cellulase (E.C. 3.2.1.91)
3. β-glucosidase or cellobiase (E.C. 3.2.1.21)

Unlike noncomplexed fungal cellulase, anaerobic bacteria possess complexed cellulase systems,

called cellulosomes (Doi and Tamaru 2001; Demain et al. 2005). The functional unit of cellulosome is scaffoldin, which contains cohesins, a cellulose-binding domain (CBD) or CBM; a dockerin, X modules of unknown function and an S-layer homology (SLH) module (Doi and Kosugi 2004). The cohesins are modules made up of ~150 amino acid residues and usually present as tandem repeats in scaffoldins. It has been demonstrated that the cohesins specifically show the interaction to the noncatalytic dockerin modules identified in cellulosomal complex (B´eguin et al. 1990, 1994; Ding et al. 2008; Fontes and Gilbert 2010). While dockerins consist of approximately 70 amino acids containing two duplicated segments (~22 amino acid residues). Dockerins are usually present in a single copy at the C terminus of cellulosomal enzymes. The first 12 amino acid residues in each segment resemble the calcium-binding loop of EF-hand motifs (helix-loop-helix motif) in which the calcium-binding residues, aspartate or asparagine, are highly conserved (Fontes and Gilbert 2010). These enzymatic subunits are bound to the scaffoldin through the interaction of the cohesins and dockerins to form the cellulosomes. The arrangement of the modules on the scaffoldin subunit and the specificity of the cohesin(s) and/or dockerin for their modular counterpart dictate the overall architecture of the cellulosome. This interaction (cohesion- dockerin) is species specific, i.e. the dockerins that are found in *Clostridium cellulolyticum* cellulosomal enzymes do not show interaction with the cohesins that are found in *C. thermocellum* and vice versa (Pages et al. 1997). Moreover, both cohesins and dockerins are highly homologous within the same species, and the residues directly involved in protein: protein recognition are highly conserved within a species.

Mechanism of Cellulases

As discussed in previous section, the structure and function of fungal and bacterial cellulases are quite different. The fungal cellulase system contains three major enzyme components: endoglucanase, cellobiohydrolase and β-glucosidase. The exoglucanase acts on the reducing ends of the cellulose chain and release cellobiose as the end product; endoglucanase randomly attacks the internal o-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glucosidases act specifically on the β-cellobiose disaccharides and produce glucose (Beguin and Aubert 1994; Kuhad et al. 1997, 2010a, b, c) (Fig. 6.1).

There is a high degree of synergy between cellobiohydrolases (exoglucanases) and endoglucanases, which is required for the efficient hydrolysis of cellulose (Din et al. 1994; Teeri et al. 1998; Boraston et al. 2004; Gupta et al. 2009). The products of endoglucanases and cellobiohydrolases, which are cellodextrans and cellobiose, respectively, are inhibitory to the enzyme's activity. Thus, efficient cellulose hydrolysis requires the presence of β-glucosidases which cleaves the final glycosidic bonds producing glucose (end product). Typically, cellobiose and cellodextrins are taken up by the microorganism and internally cleaved via cellodextrin phosphorylases or cellobiose phosphorylases to create glucose monophosphate, which is energetically favoured. Some bacteria also produce intra- or extracellular β-glucosidases to cleave cellobiose and cellodextrins and produce glucose to be taken up by or assimilated by the cell. Mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi, but it is clear that anaerobic bacteria operate a different system.

The major difference between fungal enzymes and cellulosomal enzymes is that the fungal enzymes usually contain a CBM for guiding the catalytic domain to the substrate, whereas the cellulosomal enzymes carry a dockerin domain that incorporates the enzyme into the cellulosome complex. Otherwise, both the free and cellulosomal enzymes contain very similar types of catalytic domains (Bayer et al. 2004).

The cellulosomes contain substrate-binding sites, which bind the cellulosome tightly to the substrate and concentrate the hydrolytic enzymes to specific sites (Doi 2008). CBMs play a key role in the deconstruction of complex insoluble composites exemplified by the plant cell wall. Initial studies by Bayer and their colleagues (1998) showed that the CBD contained a planar

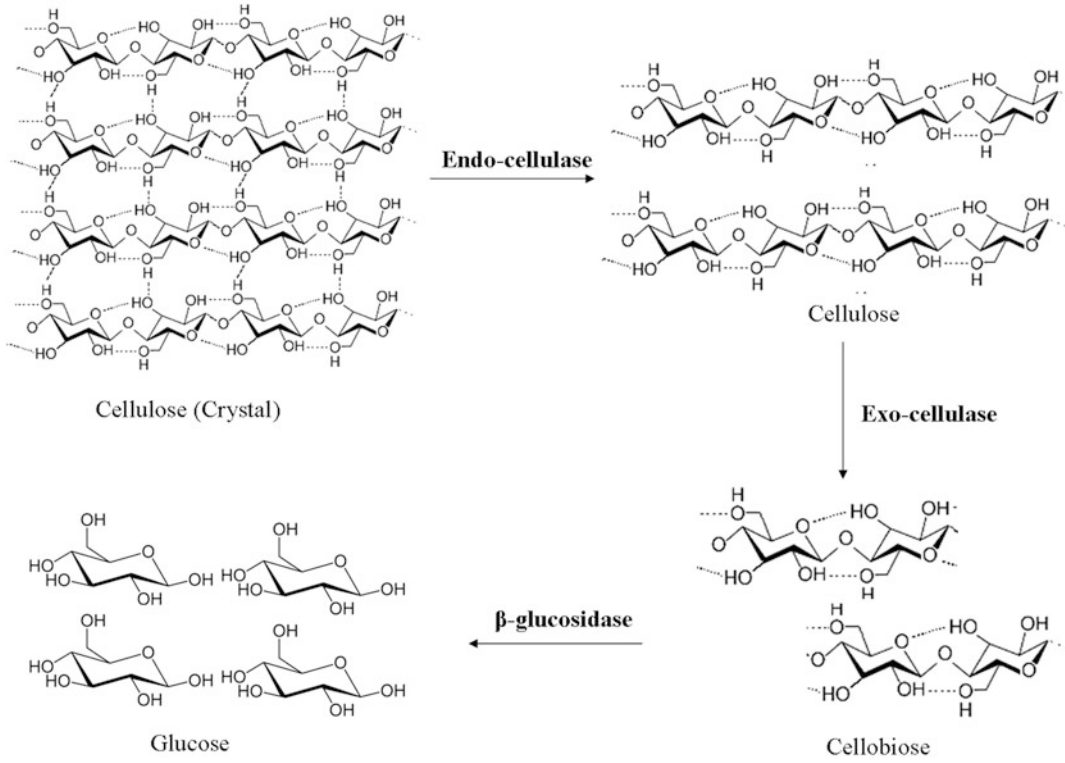

Fig. 6.1 Schematic representation of enzymatic hydrolysis of cellulose

configuration that interacted with the cellulose and involved the amino acids tryptophan, aspartic acid, histidine, tyrosine and arginine in binding the scaffoldin protein to cellulose. Upon binding to the substrate, the cellulosome complex undergoes a supramolecular reorganisation so that the cellulosomal subunits redistribute to interact with the different target substrates. For this purpose, the various cellulosomal enzymes include different types of CBMs from different families that exhibit appropriate specificities that complement the action of the parent enzyme (Bayers et al. 2004). The presence of a large variety of cellulosomal enzymes allows the cellulosome to degrade a wide variety of lignocellulosic materials (Maki et al. 2009).

Mechanistically, the reactions catalysed by all cellulases are suggested to involve general acid–base catalysis by a carboxylate pair at the enzyme active site, though different in structure. One residue acts as a general acid and protonates the oxygen of the o-glycosidic bond; at the same time, the other residue acts as a nucleophile. Depending on the distance between the two carboxylic groups, either inverting (~10 Å distances) or retaining (~5 Å-distances) mechanisms are observed in cellulases. Moreover, the involvement of multiple enzymes with a wide range of substrate specificities enables constant enzymatic actions on lignocellulosics.

Sources of Cellulases

Exploitation of cellulose depends on their efficient microbial degradation. A broad spectrum of cellulolytic microorganisms mainly fungi and bacteria have been identified over the years (Kuhad and Singh 2007). Moreover, the genetic material recovered directly from environmental samples has also shown the potential to exploit the novel cellulases trapped in the genomes of unculturable microbes. Different sources of cellulases are briefly described in this section.

Table 6.1 Cellulase-producing fungi

Microorganism	Reference	Microorganism	Reference
Acremonium cellulolyticus	Fang et al. (2008)	*Paecilomyces inflatus*	Kluczek-Turpeinen et al. (2007)
Agaricus arvensis	Jeya et al. (2010)	*Penicillium echinulatum*	Camassola and Dillon (2009)
Aspergillus niger NIAB 280	Hanif et al. (2004)	*Penicillium decumbens*	Sun et al. (2008)
Aspergillus terreus M11	Gao et al. (2008)	*Penicillium brasilianum*	Jorgensen and Olsson (2006)
Daldinia eschscholzii	Karnchanatat et al. (2008)	*Pleurotus ostreatus*	Membrillo et al. (2008)
Humicola grisea	Mello-De-Sousa et al. (2011)	*Phlebia gigantea*	Niranjane et al. (2007)
Lentinus tigrinus	Lechner and Papinutti (2006)	*Piromyces communis*	Kim et al. (2008)
Melanocarpus sp.	Kaur et al. (2006)	*Sclerotium rolfsii*	Ludwig and Haltrich (2003)
Monascus purpureus	Daroit et al. (2007)	*Scytalidium thermophilum*	Kaur et al. (2006)
Myceliophthora sp.	Badhan et al. (2007)	*Thermoascus aurantiacus*	Leite et al. (2008)
Mucor circinelloides	Saha (2004)	*Trichoderma atroviride*	Kovacs et al. (2008)
Neocallimastix frontalis	Srinivasan et al. (2001)	*Trichoderma reesei* RUT 30	Juhasz et al. (2005)
Orpinomyces sp.	Hodrova et al. (1998)		

Cellulases from Fungi

Fungi are well-known agents of decomposition of organic matter in general and of cellulosic substrate in particular; however, it is still unclear how broadly and deeply cellulolytic capability extends through the fungal world. Cellulase-producing fungi are widespread among fungi and include species from the ascomycetes (*Trichoderma reesei*), basidiomycetes (*Fomitopsis palustris*) and also few anaerobic species (*Orpinomyces* sp.) (Kuhad et al. 1994; Hodrova et al. 1998; Wyk et al. 2000; Srinivasan et al. 2001; Leite et al. 2008). Few cellulase-producing fungi are listed in Table 6.1.

Among fungi, soft rot are the best known for producing cellulases, and among them, *Trichoderma* has been the best characterised (Juhasz et al. 2005; Wen et al. 2005; Kovacs et al. 2008). Other well-known cellulase-producing soft rots are *Aspergillus niger, Fusarium oxysporum, Neurospora crassa,* etc. (Kuhad et al. 1997; Daroit et al. 2007; Gao et al. 2008; Sun et al. 2008). Besides soft rots, brown rot and white rot fungi are also actively involved in the cellulose degradation; however, both of these classes of fungi degrade wood by distinctly different mechanisms (Kuhad et al. 1994). Brown rot fungi depolymerise cellulose rapidly during the early decay of wood, and one reason may be the lack of exoglucanase (Kuhad et al. 1997). However, there are also few contrasting reports of exoglucanases-producing microbes. Recently, Deswal and coworkers (2011) have reported a brown rot fungus *Fomitopsis* sp. RCK 2010 having a good amount of all the three enzymes and have also shown the hydrolysis efficiency of pretreated lignocellulosic substrates. Besides *Fomitopsis* sp., other well-known cellulase-producing brown rots are *Poria placenta, Lenzites trabea, Coniophora puteana* and *Tyromyces palustris*. The cellulase-producing ability in white rots is heterogeneous. These microbes are most commonly known for lignin degradation. The common examples of cellulase-producing white rots are *Phanerochaete chrysosporium, Sporotrichum thermophile* and *Trametes versicolor*.

Anaerobic fungi also play a key role in the degradation of plant cell wall materials. They have the ability to degrade plant cellulose because they can produce an array of all the cellulolytic enzymes. Anaerobic fungi can only degrade the structural polysaccharides but cannot utilise the lignin moieties. Among anaerobic fungi, the most studied are *Neocallimastix frontalis* (Srinivasan et al. 2001), *Piromyces* (*Piromonas*) *communis* (Kim et al. 2008), *Orpinomyces* sp. (Hodrova et al. 1998), etc.

Table 6.2 Cellulase-producing bacteria

Source	Reference	Source	Reference
Acinetobacter junii F6-02	Lo et al. (2010)	*Butyrivibrio fibrisolvens A 46*	Hazlewood et al. (1990)
Anoxybacillus sp. *527*	Liang et al. (2009)	*Cellulomonas ANS-NS2*	Lo et al. (2009)
Acinetobacter anitratus	Ekperigin (2007)	*Cellulomonas biazotea*	Rajoka and Malik (1997)
Bacillus subtilis	Heck et al. (2002) and Kim et al. (2009)	*Clostridium thermocellum*	Chinn et al. (2008) and Dharmagadda et al. (2010)
Bacillus subtilis CBTTK 106	Krishna (1999)	*Clostridium cellulolyticum*	Desvaux et al. (2000)
Bacillus pumilus EB3	Ariffin et al. (2008)	*Clostridium acetobutylium*	Sabathe et al. (2002)
Bacillus amyloliquefaciens DL-3	Lee et al. (2008)	*Clostridium papyrosolvens*	Thirumale et al. (2001)
Bacillus licheniformis	Bischoff et al. (2006)	*Eubacterium cellulosolvens*	Moon and Anderson (2001)
Bacillus sp. *AC-1*	Li et al. (2008)	*Fibrobacter succinogenes S 85*	Bera-Maillet et al. (2009)
Bacillus sp. *DUSELR 13*	Rastogi et al. (2010)	*Geobacillus* sp. *WSUCF1*	Rastogi et al. (2010)
Bacillus circulans	Hakamada et al. (2002)	*Paenibacillus curdlanolyticus*	Waeonukul et al. (2009)
Bacillus flexus	Trivedi et al. (2011)	*Salinivibrio* sp. *NTU-05*	Wang et al. (2009)
Bacteroides sp.*P-1*	Ponpium et al. (2000)	*Ruminococcus albus F-40*	Ohara et al. (2000)

Bacteria

Cellulolytic bacteria often produce cellulases in small amounts, and degradation of cellulose seems to take place by a cluster of multienzyme complexes, which are difficult to disrupt without the loss of total activity (Kuhad et al. 1997; Doi 2008). Most of the bacterial cellulolytic enzymes are reported from *Bacillus* (Lee et al. 2008; Ariffin et al. 2008; Rastogi et al. 2010), *Acinetobacter* (Ekperigin et al. 2007; Lo et al. 2010), *Cellulomonas* (Rajoka and Malik 1997; Lo et al. 2009) and *Clostridium* (Chinn et al. 2008; Desvanux et al. 2000; Dharmagadda et al. 2010). Typically, aerobic bacteria play predominant roles in natural systems, accounting for 90–95% of bacterial cellulose degradation, the remaining 10% or less is degraded by diverse bacteria under anaerobic conditions (Carere et al. 2008). In addition to these, rumen bacteria have also shown to be producers of cellulase enzymes that can degrade structural components of cell walls (Kuhad et al. 1994). Among these, *Fibrobacter succinogenes* (Bera-Maillet et al. 2009) and *Ruminococcus albus* (Ohara et al. 2000) are most extensively studied. Recently, cellulolytic activity has been reported from thermophilic bacteria *Anoxybacillus* sp. (Liang et al. 2009), *Bacillus* sp. (Rastogi et al. 2010), *Geobacillus* sp. (Rastogi et al. 2010) and *Bacteroides* sp. (Ponpium et al. 2000). The list of few cellulase-producing bacteria is shown in Table 6.2.

Metagenomic Cellulolytic Genes

In addition to the culturable microbes, several metagenomic studies have also been carried out for the isolation of cellulase gene from various environmental samples (Ferrer et al. 2005; Palackal et al. 2007; Duan et al. 2009; Liu et al. 2009; Shedova et al. 2009; Wang et al. 2009). Ferrer et al. (2005) isolated seven new clones encoding β-1, 4-endoglucanase activity from cow rumen. Pottkamper et al. (2009) identified three novel cellulases that can degrade cellulose even in the presence of ionic liquids. Duan and coworkers (2009) isolated a novel endoglucanase C67-1, gene from buffalo rumen, which is very stable under both acidic (up to pH 3.5) and alkaline (up to pH 10.5) conditions. In another report, an endoglucanase Umcel5G, derived from rabbit cecum, was isolated which has the property to hydrolyse a wide range of substrates (Feng et al. 2007). Few studies on isolation of cellulase gene from metagenomic approaches are listed in Table 6.3.

Table 6.3 List of metagenomic sources of cellulases

Cellulase type	Source	Library type	Insert (kb)	Substrate	Reference
Endoglucanase	Anaerobic digester	Plasmid	12-Feb	CMC,MUC	Healy et al. (1995)
Endoglucanase	Lake sediment	λ phage	10-Feb	CMC	Rees et al. (2003)
Endoglucanase	Soil	Cosmid	25–40	CMC	Voget et al. (2003)
Endoglucanase	Lake sediment	λ phage	2.0–5.5	CMC	Grant et al. (2004)
Endoglucanase	Cow rumen	λ phage	5.5	OBR-HEC	Ferrer et al. (2005)
β-glucosidase	Soils from wetland	Fosmid	35	MUC	Kim et al. (2007)
Endoglucanase	Rumen fluid	λ phage	3	Dye-linked azo-xylan	Palackal et al. (2007)
Endoglucanase/β-glucosidase	Rabbit cecum	Cosmid	35.1	CMC,MUC, EH-FAC	Feng et al. (2007)
Endoglucanase	Hindgut of higher termite	Fosmid and plasmid	–	PASC	Warnecke et al. (2007)
Endoglucanase	Soil	Fosmid	–	CMC	Kim et al. (2008)
Endoglucanase/β-glucosidase	Soil, rumen	λ phage	5.3	CMC,MUC, EH-FAC	Wang et al. (2009)
Endoglucanase	Cow rumen	Plasmid	15	CMC	Shedova et al. (2009)
Endoglucanase/β-glucosidase	Compost	Cosmid	33	CMC	Pang et al. (2009)
Endoglucanase/β-glucosidase	Buffalo rumen	Cosmid	35	CMC,MUC, EH-FAC	Duan et al. (2009)
Endoglucanase/β-glucosidase/ Cellodextrinase					
Endoglucanase	Buffalo rumen	Cosmid	46.1	MUC	Liu et al. (2009)
β-glucosidase	Alkaline polluted soil	Plasmid	3.5	EH-FAC	Jiang et al. (2009)
Endoglucanase	Aquatic community and soil	Cosmid	–	CMC	Pottka¨mper et al. (2009)
β-glucosidase	Sludge	Cosmid	35	EH-FAC	Jiang et al. (2010)
Endoglucanase	Pot soil	Fosmid	40	CMC	Sita (2010)

Industrial Application of Cellulases

Cellulases have biotechnological potential in various industries, including food, brewery and wine, industrial waste to chemical feedstock, animal feed, textile and laundry, pulp and paper and agriculture, as well as in research and development of single-cell protein (Poutanen 1997; Bhat and Bhat 1997; Bajpai 1999; Bergqvist et al. 2005; Bamforth 2009; Kuhad et al. 2011).

Role of Cellulases in Food Industry

Cellulases play a prominent role in extraction of juice from a wide range of fruits and vegetables (Humpf and Schrier 1991; Sreenath et al. 1994; Bhat 2000; Bergqvist et al. 2005; Kuhad et al. 2011) (Table 6.4). Cellulases are used not only to improve the cloud stability and texture of nectars and purees but also to decrease their viscosity (Grassin and Fauquembergue 1996; Bhat 2000; Hui 2006). Cellulases are also used for food colouring agents production and in the extraction of olive oil and carotenoids (Grohman and Baldwin 1992; Faveri et al. 2008; Belitz et al. 2009). Moreover, cellulase is also used to alter the sensory properties of fruits and vegetables, by increasing their aroma and volatile characteristics (Humpf and Schrier 1991; Krammer et al. 1991; Dauty 1995; Bhat 2000; Hui 2006).

Role of Cellulases in Beer Industry

Beer brewing involves malting of the barley in a malt house followed by the preparation and fermentation of the wort in the brewery. Malting depends mainly on germination of seed, which initiates the biosynthesis and activation of

Table 6.4 Role of cellulases in food biotechnology

S. no.	Function	Application	Reference
1.	Hydrolysis of cell wall components; decreasing the viscosity and maintaining the texture of fruit juice	Improvement in pressing and extraction of juice from fruits and oil from olives; releasing flavour, enzymes, proteins, polysaccharides, starch and agar	Galante et al. (1998), Bergqvist et al. (2005), and Kuhad et al. (2011)
2.	Infusion of pectinase and glucosidase for easy peeling/firming of fruits and vegetables	Alteration of the sensory properties of fruits and vegetables	Krammer et al. (1991)
3.	Partial or complete hydrolysis of cell wall polysaccharides and substituted celluloses	Improvement in soaking efficiency; homogeneous water absorption by cereals; the nutritive quality of fermented foods; the rehydrability of dried vegetables and soups; the production of oligosaccharides as functional food ingredients and low-calorie food substituents and biomass conversion; extract of olive oil, Purees	Beguin and Aubert (1994), Bhat and Bhat (1997), Cinar (2005), and Faveri et al. (2008)
4.	Hydrolysis of arabinoxylan and starch	Separation and isolation of starch and gluten from wheat flour	Bhat (2000)
5.	Release of antioxidants from fruit and vegetable pomace	Controlling coronary heart disease and atherosclerosis; reducing food spoilage	Bhat (2000)

amylases, carboxypeptidase and cellulases which act in synergy under optimal conditions to produce high-quality malt. Therefore, the addition of cellulases is known to improve not only the beer qualities but also their overall production efficiency (Galante et al. 1998).

Role of Cellulases in Animal Feed Industry

Cellulases have a wide range of potential applications in animal feeding (Lewis et al. 1996; Bhat 2000; Knowlaton et al. 2007; Pariza and Cook 2010). Cellulases are the main class of enzymes used in monogastric feed and ruminant feed (Graham and Balnave 1995; Lewis et al. 1996; Kung et al. 1997). They can be used either to eliminate anti-nutritional factors present in raw materials or to degrade certain cereal components in order to improve the nutritional value of feed.

Role of Cellulases in Textile and Laundry Industry

The cellulases in textile industry are most commonly used for biostoning, biopolishing and biofinishing (Kirk et al. 2002; Lima et al. 2005; Ibrahima et al. 2010). The advantages of using cellulase-based biostoning are less labour-intensive, worn look, reduce damage, and create the possibility to automate the process (Galante et al. 1998; Pazarlioglu et al. 2005). While during biopolishing, the cellulases act on small fibre ends that protrude from the fabric surface, where the mechanical action removes these fibres and polishes the fabrics (Sukumaran et al. 2005). The cellulases remove short fibres and surface fuzziness, smoothen the appearance, remove the soil, improve colour brightness and increase hydrophilicity and moisture absorbance (Sukumaran et al. 2005; http://www.mapsenzymes.wm/enzymes_detergent.asp).

Role of Cellulases in Pulp and Paper Industry

Cellulase has been used in the pulp and paper industry for various purposes. The effect of enzymatic modification of coarse mechanical pulp using cellulase led to significant energy saving (Pere et al. 1996). Cellulases have also been used for the modification of fibre properties to improve drainage, beatability and runnability of the paper industry (Noe et al. 1986; Pommier et al. 1989, 1990). The cellulases have also been observed to

be the most effective for recycling the waste papers from books, magazines and newspaper which could have value addition via deinking and reuse of fibre either in manufacturing of newspaper or ethanol production (Kuhad et al. 2010a, b, c). The main advantage of enzymatic deinking is the avoidance of the use of alkali. Deinking, using enzymes at acidic pH, also prevents the alkaline yellowing, simplifies the deinking process, changes the ink particle size distribution and reduces the environmental pollution (Kirk et al. 2002; Kuhad et al. 2010a, b, c; Liu et al. 2010). In addition, the enzymatic deinking improves the fibre brightness, strength properties, pulp freeness and cleanliness as well as reduces fine particles in the pulp (Liu et al. 2009; Kuhad et al. 2010a, b, c).

Role of Cellulases in Agriculture Industry

Many cellulolytic fungi such as *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp. and *Penicillium* sp. are known to facilitate enhanced seed germination, rapid plant growth and flowering and increased crop yields (Bailey and Lumsden 1998; Harman and Bjorkman 1998; Bhat 2000; Fontaine et al. 2004; Wei et al. 2009). β-1,3-glucanase from *T. harzianum* CECT 2413 induced morphological changes such as hyphal tip swelling, leakage of cytoplasm and the formation of numerous septae and inhibited the growth of *Rhizopus solani* and *Fusarium* sp. (Benitez et al. 1998). Besides, they are also capable of degrading the cell wall of plant pathogens and controlling the plant disease. Cellulase is also used to improve soil quality and reduce dependence on mineral fertilisers (Escobar and Hue 2008; Han and He 2010).

Role of Cellulases in R&D Industries

Cellulases and related enzymes can also be used as potential tools for generating new strains capable of producing high levels of enzymes of commercial interest. Mixture of cellulases and other enzymes results in the solubilisation of fungal or plant cell wall to produce protoplast (Beguin and Aubert 1994). Cellulose-binding domains (CBD) of cellulases, which function normally when fused to heterologous proteins, have been successfully used either as an affinity tag for the purification of proteins or immobilisation of fusion proteins (Assouline et al. 1993; Greenwood et al. 1992; Tomme et al. 1995). Similarly, using the scaffoldin CBD of the *C. thermocellum* cellulosome, a novel affinity column was prepared for the purification of antibodies (Bayer et al. 1995).

Role of Cellulases in Biofuel Industry

A potential application of cellulases is the conversion of cellulosic material to glucose and other fermentable sugars, which in turn can be, used as microbial substrate for the production of single-cell protein or fermentation products like ethanol (Sukumaran et al. 2005; Kuhad et al. 2010a, b, c). Production of ethanol from renewable resources via fermentation represents an important process for production of alternative fuels (Sukumaran et al. 2005; Kuhad et al. 2010a, b, c, 2011). Ethanol has a unique combination of attributes including low life-cycle greenhouse gas emissions, a high level of sustainability, and seamless integration into the existing transport system with potential to have a large-scale impact (Ward and Singh 2002; Gupta et al. 2009; Kuhad et al. 2010a, b, c).

Cellulase in Pharmaceutical Industries

Since humans poorly digest cellulose fibre, taking a digestive enzyme product, like digestin, that contains cellulase enzymes could be important for healthy cells. Fungal hemicellulase and cellulase enzyme system helps in rapid hydrolysis of cellulose, hemicellulose and beta-glucan polymers in food. The gummy substances take up a lot of water and swell up to about ten times, thus hindering the action of enzymes on other biomolecules (http:/www.expresspharmaonline.com/20041028/biochemicals01.html).

Table 6.5 Applications of plant cell-wall-degrading enzymes and cellulolytic microorganisms in research and development as well as in agriculture

S. no.	Function	Application	Reference
1.	Solubilisation of plant or fungal cell walls	Production of plant or fungal protoplasts, hybrid and mutant strains	Beguin and Aubert (1994)
2.	Inhibition of spore germination, germ tube elongation and fungal growth	Biocontrol of plant pathogens and diseases	Lorito et al. (1994), Benitez et al. (1998), and Harman and Kubicek (1998)
3.	Affinity tag, affinity systems, conjugation and gene fusion	Affinity purification, immobilisation and fusion of proteins, enzymes and antibodies; production of hybrid molecules for various applications	Bayer et al. (1995)
4.	Exogenous cellulase accelerated decomposition of cellulose in soil	Soil fertility, plant growth	Han and He (2010)

Strategies for Cellulase Improvement

The use of cellulases for various applications demands their cost-effective production. Therefore, to improve cellulases titer and their ratios, various approaches like mutagenesis, genetic engineering and protein engineering have been used.

Mutation is one of the most commonly used approaches for the cellulase improvement (Durand et al. 1988; Anwar et al. 1996; Chand et al. 2005; Adsul et al. 2007). There are several reports where mutagenised strains have shown better properties over their parent strain. Chand et al. (2005) gave ETBr and 1-methyl −3-nitro-1-nitrosoguanidine treatment to *A. niger,* and the resultant strain *A. niger* CMV5-A10 exhibited twofold enhanced cellulase production. Similarly Adsul et al. (2007) increased the cellulase production twofold from EMS and UV-mutated *P. janthinellum* NCIM 1171. Though mutagenesis has improved the cellulase quality, but the instability of mutants due to reversion remains a big hurdle.

Moreover, cloning and expression of both bacterial and fungal cellulase genes in various hosts have also been attempted to improve the cellulase production (Kataeva et al. 1999; Abdeev et al. 2003; Park et al. 2005; Hong et al. 2009; Mekoo et al. 2010) (Table 6.5). Cloning of cellulases (endoglucanase and cellobiohydrolase) from *Clostridium* has been reported by several workers (Shima et al. 1989; Wang et al. 1993). A hyperthermophile cellulase from *Pyrococcus horikoshii* was successfully cloned and overexpressed in the *B. brevis* host vector system and enhanced the cellulase production by 20-fold (Kashima and Udaka 2004). Similarly, Park and coworkers (2005) have cloned a thermostable exoglucanase gene from *Streptomyces* sp. M23 in *S. lividans* TK-24 which was stable up to 100°C. In another report, Li et al. (2008) cloned a thermostable endoglucanase gene from *B. subtilis* in *E. coli* successfully with threefold increase in activity. While recently a novel, acid-tolerant endoglucanase from *Martelella mediterranea* a marine bacterium cloned and expressed in *E. coli* with unchanged properties (Table 6.6).

Similar to bacterial cellulases, cloning of fungal cellulases and expression in appropriate host have also been carried out since long (Table 6.5). Hamada and Hirohashi (2000) successfully cloned and characterised the exocellulase gene from white rot fungus *Irpex lacteus* using northern hybridisation. Haakana et al. (2004) cloned three genes (two endoglucanase and one CBH) from *Melanocarpus albomyces* and expressed in *T. reesei* under the control of the *T. reesei* CBHI promoter increasing the production level several times. While Hong et al. (2007) reported cloning of thermostable β-glucosidase from *T. aurantiacus* and expressed the β-glucosidase gene in *Pichia pastoris* and as a result, they developed recombinant yeast strain able to utilise cellobiose as a carbon source. Further, to improve the β-glucosidase yield and total cellulase activity of *T. reesei*,

Table 6.6 List of some recombinant cellulase-producing microorganisms

Microorganisms	Type	Cloning/expression vector	Cloning host	Reference
A. Bacteria				
Pectobacterium chrysanthemi	Glycosyl hydrolase	pBluescript II SK +	*E. coli*	Cho et al. (2002)
Sinorhizobium meliloti	CMCase	pUC 18, pet 22b	*E. coli*	Michaud et al. (2002)
Clostridium thermocellum	Endoglucanase	E35S-L-lic B	*Tobacco*	Abdeev et al. (2003)
Bacillus licheniformis	Endoglucanase	pBluescript SK(+)	*E. coli*	Liu et al. (2004)
Xylella fastidiosa	Endoglucanase	pet 20(b)	*E. coli*	Wulff et al. (2006)
Pseudomonas DY 3	–	pGEMT	*E. coli*	Zeng et al. (2006)
Cytophaga hutchinsonii	Endoglucanase	pGEM/pet 28 a	*E. coli XLB- Gold*	Louime et al. (2007)
Bacillus subtilis	Endocellulase	pGEMT/pet 28 a	*E. coli*	Li et al. (2008)
Myxobacter sp. *AL-1*	Cellobiohydrolase	pCR-Blunt II-TOPO	*E. coli*	Ramírez et al. (2008)
Bacillus subtilis	Cel L 15, Cel L73	pet 28 a	*E. coli*	Li et al. (2009)
Caldicellulosiruptor saccharolyticus	β-glucosidase	pet 28 a	*E. coli ER 2566*	Hong et al. (2009)
Martelella mediterranea	Endoglucanase	pUC 18/pGEX-6p-1	*E. coli*	Dong et al. (2010)
B. Fungi				
Thermoascus aurantiacus	CBH	λgt10 vector	*S. cerevisiae*	Hong et al. (2003)
Aspergillus aculeatus	Cellobiohydrolase		*A. oryzae*	Kanamasa et al. (2003)
Thermobifida fusca	Endoglucanase	pIJ699	*S. lividans*	Posta et al. (2004)
Melanocarpus albomyces	Endoglucanase	pALK1231	*T. reesei*	Haakana et al. (2004)
Talaromyces emersonii	β-glucosidase	lGEM-11	*E. coli*	Collins et al. (2007)
Penicillium chrysogenum	CBH	pGEM-T vector	*E. coli*	HOU et al. (2007)
Thermoascus aurantiacus	β-glucosidase	pPICZαvector	*P. pastoris*	Hong et al. (2007)
Irpex lacteus	Cellobiohydrolase	pUC119/PT7-Blue	*E. coli*	Toda et al. (2008)
Rhizopus stolonifer	CMCase	–	*E. coli*	Tang et al. (2009)
Chaetomium thermophilum	Cellobiohydrolase	–	*P. pastoris*	Li et al. (2008)
Penicillium sp.	Endoglucanase	pJAL721	*A. oryzae*	Krogh et al. (2009)
Neocallimastix sp.		pCT/pTRW10	*Lactococcus lactis*	Ozkose et al. (2009)
Penicillium echinulatum	Endoglucanase	pPIC9	*P. pastoris*	Rubini et al. (2009)
Oenococcus oeni	Phosphoglucosidase	pet 14 b	*E. coli*	Capaldo et al. (2011)
Penicillium decumbens	Endoglucanase	pMD18-T/ pAJ401	*S. cerevisiae*	Xiao- Min et al. (2010)
Trichoderma reesei	CBH,	pMI519	*Ashbya gossypii*	Ribeiro et al. (2010)
Trichoderma reesei	β-glucosidase, CBH	pMD18-T	*T. reesei*	Zhang et al. (2010)
Penicillium occitanis	CBH	*pMOSblue T-vector*	*Penicillium occitanis*	Bhiri et al. (2010)

extracellular β-glucosidase was overexpressed under the control of the modified four-copy CBHI promoter (Zhang et al. 2010).

In addition to genetic engineering strategies, protein-engineering approaches have also been used to improve cellulase quality. Escover-Kousen et al. (2004) observed 40% increase in cellulase activity on amorphous cellulose or soluble cellulose using integration of computer modelling and site-directed mutagenesis. Moreover, by combining 2 CBDs, one from *T. reesei* and other from *C. stercorarium,* Mahadeven et al. (2008) increased the activity by 14–18 folds. Recently, Scott et al. (2010) modified the linker peptides of cellulase to reduce its binding to lignin for enhanced cellulose hydrolysis.

Conclusion

In a world with a rapidly increasing population and approaching exhaustion of many natural resources, enzyme technology offers a great potential for many industries to help meet the challenges they will face in years to come. As outlined above, cellulases are used in several different industrial products and processes, and new areas of application are constantly being added. The use of recombinant gene technology has further improved manufacturing processes and enabled the commercialisation of enzymes that could previously not be produced. Furthermore, the latest developments within modern biotechnology, introducing protein engineering and directed evolution, have further revolutionised the development of industrial enzymes, which are opening new avenues for utilisation of various agrowastes as a source of renewable resources and could solve the problem of waste management as well.

References

Abdeev RM, Goldenkov IVA, Musiychuk KA, Piruzian ES (2003) Expression of a thermostable bacterial cellulase in transgenic tobacco plants. Russ J Genet 39:300–305

Adsul MG, Bastawde KB, Varma AJ, Gokhle DV (2007) Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. Bioresour Technol 98:1467–1473

Anwar MN, Suto M, Tomita F (1996) Isolation of mutants of *Penicillium purpurogenum* resistant to catabolite repression. Appl Microbiol Biotechnol 45:684–687

Ariffin H, Hassan M, Shah UKM, Abdullah N, Ghazali FM, Shirai Y (2008) Production of bacterial endoglucanase from pretreated oil palm empty fruit bunch by *Bacillus pumilus* EB3. J Biosci Bioeng 106:231–236

Assouline Z, Shen H, Kilburn DG, Warren RAJ (1993) Production and properties of a factor X-cellulose-binding domain fusion protein. Protein Eng 6:787–792

Badhan AK, Chadha BS, Kaur J, Saini HS, Bhat MK (2007) Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099. Bioresour Technol 98:504–510

Bailey BA, Lumsden RD (1998) Direct effects of *Trichoderma* and *Gliocladium* on plant growth and resistance to pathogens. In: Harman GF, Kubicek CP (eds) *Trichoderma & Gliocladium*—Enzymes, biological control and commercial applications, vol 2. Taylor & Francis, London, pp 327–342

Bajpai P (1999) Applications of enzymes in the pulp and paper industry. Biotechnol Prog 15:147–157

Bamforth CW (2009) Current perspectives on the role of enzymes in brewing. J Cereal Sci 50:353–357

Bayer EA, Morag E, Wilchek M, Lamed R, Yaron S, Shoham Y (1995) Cellulosome domains for novel biotechnological application. In: Petersen SB, Svensson B, Pedersen S (eds) Carbohydrate bioengineering. Progress in biotechnology, vol 10. Elsevier, Amsterdam, pp 251–260

Bayer EA, Chanzy H, Lamed R, Shoham Y (1998) Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 8:548–557

Bayer EA, Belaich JP, Shoham Y, Lamed R (2004) The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 58:521–554

Beguin P, Aubert JP (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13:25–58

Belitz HD, Grosch W, Schieberle P (2009) Springer. Food chemistry 4th revised and extended edition. Annu Rev Biochem 79:655–681

Benitez T, Limon C, Delgado-Jarana J, Rey M (1998) Glucanolytic and other enzymes and their genes. In: Harman GF, Kubicek CP (eds) *Trichoderma & Gliocladium*—Enzymes, biological control and commercial applications, vol 2. Taylor &Francis, London, pp 101–127

Bera-Maillet C, Mosoni P, Kwasiborski A, Suau F, Ribot Y, Forano E (2009) Development of a RT-qPCR method for the quantification of *Fibrobacter succinogenes* S85 glycoside hydrolase transcripts in the rumen content of gnotobiotic and conventional sheep. J Microbiol Methods 77:8–16

Bergqvist SW, Sandberg A-S, Carlsson N-G, Andlid T (2005) Improved iron solubility in carrot juice fermented by homo- and hetero-fermentative lactic acid bacteria. Food Microbiol 22:53–61

Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383

Bhat MK, Bhat S (1997) Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 15:583–620

Bhiri F, Gargouri A, Ali MB, Belghith H, Blibech M, Chaabouni SE (2010) Molecular cloning, gene expression analysis and structural modelling of the Cellobiohydrolase I from *Penicillium occitanis*. Enzyme Microb Technol 46:74–81

Bischoff KM, Rooney AP, Li X, Liu S, Hughes SR (2006) Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. Biotechnol Lett 28: 1761–1765

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate- binding modules: fine tuning polysaccharide recognition. Biochem J 382:769–781

Camassola M, Dillon AJP (2009) Biological pretreatment of sugarcane bagasse for the production of cellulases and xylanases by *Penicillium echinulatum*. Ind Crop Prod 29:642–647

Capaldo A, Walker ME, Ford CM, Jiranek V (2011) β-Glucoside metabolism in *Oenococcus oeni*: cloning and characterization of the phospho-b-glucosidase bglD. Food Chem 125:476–482

Carere CR, Sparling R, Cicek N, Levin DB (2008) Third generation biofuels via direct cellulose fermentation. Int J Mol Sci 9:1342–1360

Chand P, Aruna A, Maqsood AM, Rao LV (2005) Novel mutation method for increased cellulase production. J Appl Microbiol 98:318–323

Chinn MS, Nokes SE, Strobel HJ (2008) Influence of moisture content and cultivation duration on *Clostridium thermocellum* 27405 end-product formation in solid substrate cultivation on Avicel. Bioresour Technol 99:2664–2671

Cho SJ, Park SR, Kim MK, Lim WJ, Ryu SK, An CL, Hong SY, Kim H, Cho YU, Yun HD (2002) Cloning of the *cel*8Y gene from *Pectobacterium chrysanthemi* PY35 and its comparison to *cel* genes of soft-rot *Pectobacterium*. Mol Cell 13:28–34

Cinar I (2005) Effects of cellulase and pectinase concentrations on the colour yield of enzyme extracted plant carotenoids. Process Biochem 40:945–949

Collins CM, Murray PG, Denman S, Morrissey JP, Byrnes I, Teeri TT, Tuohy MG (2007) Molecular cloning and expression analysis of two distinct b-glucosidase genes, bg1 and aven1, with very different biological roles from the thermophilic, saprophytic fungus *Talaromyces emersonii*. Mycol Res 111:840–849

Daroit DJ, Silveria ST, Hertz PF, Brandelli A (2007) Production of extracellular β-glucosidase by *Monascus purpureus* on different growth substrates. Process Biochem 42:904–908

Dauty ME (1995) Fruit and vegetables processing, FAO agriculture services bulletins 119. FAO, Rome

Davison A, Blaxter M (2005) Ancient origin of glycosyl hydrolase family 9 cellulase genes. Mol Biol Evol 22:1273–1284

Demain AL, Newcomb M, Wu JH (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69(1):124–154

Desvaux ML, Guedon E, Petitdemange H (2000) Cellulose catabolism by *Clostridium cellulolyticum* growing in batch culture on defined medium. Appl Environ Microbiol 66:2461–2470

Deswal D, Khasa YP, Kuhad RC (2011) Optimization of cellulase production by brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. Bioresour Technol 102:6065–6072

Dharmagadda VSS, Nokes SE, Strobel HJ, Flythe MD (2010) Investigation of the metabolic inhibition observed in solid-substrate cultivation of *Clostridium thermocellum* on cellulose. Bioresour Technol 101: 6039–6044

Ding S, Xu Q, Crowley M, Zeng Y, Nimlos M, Lamed R, Bayer EA, Himmel ME (2008) A biophysical perspective on the cellulosome: new opportunities for biomass conversion. Curr Opin Biotechnol 19:218–227

Doi RH (2008) Cellulases of mesophilic microorganisms. Ann N Y Acad Sci 1125:267–279

Doi RH, Tamaru Y (2001) The Clostridium cellulovorans cellulosome: an enzyme complex with plant cell wall degrading activity. Chem Rec 1:24–32

Doi RH, Kosugi A (2004) Cellulosomes: plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol 2:541–551

Dong J, Hong Y, Shao Z, Liu Z (2010) Molecular cloning, purification, and characterization of a novel, acidic, pH-stable endoglucanase from *Martelella mediterranea*. J Microbiol 48:393–398

Duan CJ, Xian L, Zhao GC, Feng Y, Pang H, Bai XL, Tang JL, Ma QS, Feng JX (2009) Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. J Appl Microbiol 107:245–256

Durand H, Clanet H, Tribay G (1988) Genetic improvements of Trichoderma reesei for large scale cellulase productions. Enzyme Microb Technol 10:341–346

Ekperigin MM (2007) Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. Afr J Biotechnol 6:28–33

Escobar MEO, Hue NV (2008) Temporal changes of selected chemical properties in three manure – amended soils of Hawaii. Bioresour Technol 99:8649–8654

Escovar-Kousen JM, Wilson D, Irwin D (2004) Integration of computer modeling and initial studies of site-directed mutagenesis to improve cellulase activity on Cel9A from Thermobifida fusca. Appl Biochem Biotechnol 113–116:287–297

Fang X, Yano S, Inoue H, Sawayama S (2008) Lactose enhances cellulase production by the filamentous fungus *Acremonium cellulolyticus*. J Biosci Bioeng 106:115–120

Faveri DD, Aliakbarian B, Avogadro M, Perego P, Converti A (2008) Improvement of olive oil phenolics content by means of enzyme formulations: effect of different enzyme activities and levels. Biochem Eng J 41:149–156

Feng Y, Duan CJ, Pang H, Mo XC, Wu CF, Yu Y, Hu YL, Wei J, Tang JL, Feng JX (2007) Cloning and identification of novel cellulase genes from uncultured microorganisms in rabbit cecum and characterization of the expressed cellulases. Appl Microbiol Biotechnol 75:319–328

Ferrer M, Golyshina OV, Chernikova TN, Khachane AN, Reyes-Duarte D, Santos VA, Strompl C, Elborough K (2005) Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. Environ Microbiol 7:1996–2010

Fontaine S, Bardoux G, Benest D, Verdier B, Mariotti A, Abdadie L (2004) Mechanisms of the priming effect in the Savannah soil amended with cellulose. Soil Sci Soc Am J 68:125–131

Fontes CMGA, Gilbert HJ (2010) Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. Annu Rev Biochem 79:655–681

Galante YM, De Conti A, Monteverdi R (1998) Application of Trichoderma enzymes in food and feed industries. In: Harman GF, Kubicek CP (eds)

Trichoderma & Gliocladium—Enzymes, biological control and commercial applications, vol 2. Taylor & Francis, London, pp 327–342

Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour Technol 99:7623–7629

Graham H, Balnave D (1995) Dietary enzymes for increasing energy availability. In: Wallace RJ, Chesson A (eds) Biotechnology in animal feeds and animal feedings. VHC, Weinheim, pp 296–309

Grant S, Sorokin DY, Grant WD, Jones BE, Heaphy S (2004) A phylogenetic analysis of Wadi el Natrun soda lake cellulase enrichment cultures and identification of cellulase genes from these cultures. Extremophiles 8:421–429

Grassin C, Fauquembergue P (1996) Wine. In: Godfrey T, West S (eds) Industrial enzymology, 2nd edn. Macmillan Press, London, pp 374–383

Greenwood JM, Ong E, Gilkes NR, Antony R, Warren J, Miller RC Jr, Kilburn DG (1992) Cellulose-binding domains: potential for purification of complex proteins. Protein Eng 5:361–365

Grohman K, Baldwin EA (1992) Hydrolysis of orange peel with pectinase and cellulase enzymes. Biotechnol Lett 14:1169–1174

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresour Technol 100:1214–1220

Haakana H, Miettinen-Oinonen A, Joutsjoki V, Mäntyl A, Suominen P, Vehmaanper J (2004) Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei*. Enzyme Microb Technol 34:159–167

Hakamada Y, Endo K, Takizawa S, Kobayashi T, Yamane T, Ito S (2002) Enzymatic properties, crystallization, and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. Biochim Biophys Acta 1570:174–180

Hamada N, Hirohashi K (2000) Cloning and transcriptional analysis of Exocellulase I gene from Irpex lacteus. J Tokyo Univ Fish 87:39–44

Han W, He M (2010) The application of exogenous cellulase to improve soil fertility and plant growth due to acceleration of straw decomposition. Bioresour Technol 101:3724–3731

Hanif A, Yasmeen A, Rajoka MJ (2004) Induction, production, repression and de-repression of exoglucanase synthesis in *Aspergillus niger*. Bioresour Technol 94:311–319

Harman GE, Bjorkman T (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. *Trichoderma* and *Gliocladium*: enzymes, biological control and commercial applications, vol 2. Taylor & Francis Ltd, London, pp 229–265

Hazlewood GP, Davidson K, Laurie JI, Romanie MPM, Gilbert HJ (1990) Cloning and sequencing of the ceZA gene encoding endoglucanase A of *Butyvivibvio jibrisolvens* strain A46. J Gen Microbiol 136:2089–2097

Healy FG, Ray RM, Aldrich HC, Wilkie AC, Ingram LO, Shanmugam KT (1995) Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. Appl Microbiol Biotechnol 43:667–674

Heck JX, Hertz PF, Ayub MAZ (2002) Cellulase and xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based Solid-State cultivation. Braz J Microbiol 33:213–218

Hodrova B, Kopecny J, Kas J (1998) Cellulolytic enzymes of rumen anaerobic fungi *Orpinomyces joyonii* and *Caecomyces communis*. Res Microbiol 149:417–427

Hong J, Tamak H, Yamamoto IK, Kumagai H (2003) Cloning of a gene encoding thermostable cellobiohydrolase from *Thermoascus aurantiacus* and its expression in yeast. Appl Microbiol Biotechnol 63:42–50

Hong J, Tamak H, Yamamoto IK, Kumagai H (2007) Cloning and functional expression of thermostable β-glucosidase gene from *Thermoascus aurantiacus*. Appl Microbiol Biotechnol 73:1331–1339

Hong MR, Kim YS, Park CS, Lee JK, Kim YS, Oh DK (2009) Characterization of a recombinant β-glucosidase from the thermophilic bacterium *Caldicellulosiruptor saccharolyticus*. J Biosci Bioeng 108:36–40

Hou Y, Wang T, Long H, Zhu H (2007) Cloning, sequencing and expression analysis of the first cellulase gene encoding cellobiohydrolase 1 from a cold-adaptive *Penicillium chrysogenum* FS010. Acta Biochim Biophys Sin 39:101–107

Hui YH (2006) Handbook of fruits and fruit processing. Blackwell, Iowa

Humpf H-U, Schrier P (1991) Bound aroma compounds from the fruit and the leaves of blackberry (*Rubus laciniata* L.). Agric Food Chem 39:1830–1832

Ibrahima NA, EL-Badry K, Eid BM, Hassan TM (2010) A new approach for biofinishing of cellulose-containing fabrics using acid cellulases. Carbohydr Polym 83:116–121

Jagtap S, Rao M (2005) Purification and properties of a low molecular weight 1,4-beta-d-glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic *Thermomonospora* sp. Biochem Biophys Resour Commu n 329(1):111–116

Jeya M, Nguyen N, Moon H, Kim S, Lee J (2010) Conversion of woody biomass into fermentable sugars by cellulase from *Agaricus arvensis*. Bioresour Technol 101:8742–8749

Jiang C, Ma G, Li S, Hu T, Che Z, Shen P, Yan B, Wu B (2009) Characterization of a novel beta-glucosidase-like activity from a soil metagenome. J Microbiol 47:542–548

Jiang C, Hao ZY, Jin K, Li SX, Che ZQ, Ma GF, Wu B (2010) Identification of a metagenome-derived b-glucosidase from bioreactor contents. J Mol Catal B: Enzym 63:11–16

Jorgensen H, Olsson L (2006) Production of cellulases by *Penicillium brasilianum* IBT 20888—Effect of

substrate on hydrolytic performance. Enzyme Microb Technol 38:381–390

Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L (2005) Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem 40:3s519–3525

Kanamasa S, Mochizuki M, Takada G, Kawaguchi T, Sumitani JI, Arai M (2003) Overexpression of *Aspergillus aculeatus* Cellobiohydrolase I gene in *Aspergillus oryzea*. J Biosci Bioeng 95(6):627–629

Karnchanatat A, Petsom A, Sangvanich P, Piapukiew J, Whalley AJS, Reynolds CD, Gadd GM, Sihanonth P (2008) A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. Enzyme Microb Technol 42:404–413

Kashima Y, Udaka S (2004) High level production of hyperthermophilic cellulase in Bacillus brevis. Biosci Biotechnol Biochem 68:235–237

Kataeva I, Li XL, Chen H, Choi SK, Ljungdahi LG (1999) Cloning and sequence analysis of a new cellulase gene encoding CelK, a major cellulosome component of *Clostridium thermocellum*: evidence for gene duplication and recombination. J Bacteriol 181:5288–5295

Kaur J, Chadha BS, Saini HS (2006) Regulation of cellulase production in two thermophilic fungi Melanocarpus sp. MTCC 3922 and *Scytalidium thermophilum* MTCC 4520. Enzyme Microb Technol 38:931–936

Kim SJ, Lee CM, Kim MY, Yeo YS, Yoon SH, Kang HC, Koo BS (2007) Screening and characterization of an enzyme with beta-glucosidase activity from environmental DNA. J Microbiol Biotechnol 17:905–912

Kim SJ, Lee CM, Han BR, Kim MY, Yeo YS, Yoon SH, Koo BS, Jun HK (2008) Characterization of a gene encoding cellulase from uncultured soil bacteria. FEMS Microbiol Lett 282:44–51

Kim B, Lee B, Lee Y, Jina I, Chung C, Lee J (2009) Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53. Enzyme Microb Technol 44:411–416

Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351

Kluczek-Turpeinen B, Maijala P, Hofrichter M, Hatakka A (2007) Degradation and enzymatic activities of three *Paecilomyces inflatus* strains grown on diverse lignocellulosic substrates. Int Biodeter Biodegr 59:283–291

Knowlaton KF, Taylor MS, Hill SR, Cobb C, Wilson KF (2007) Manure nutrient excretion by lactating cows fed endogenous phytase and cellulase. J Dairy Sci 90:4356–4360

Kovacs K, Megyeri L, Szakacs G, Kubicek CP, Galbe M, Zacchi G (2008) *Trichoderma atroviride* mutants with enhanced production of cellulase and β-glucosidase on pretreated willow. Enzyme Microb Technol 43:48–55

Krammer G, Winterhalter P, Schwab M, Schrier P (1991) Glycosidically bound aroma compounds in the fruits of *Prunus* species: apricot (*P. armeniaca*, L.), Peach (*P. persica,* L.), Yellow plum (*P. domestica*, L. ssp. Syriaca). J Agric Food Chem 39:778–781

Krishna C (1999) Production of bacterial cellulases by solid state bioprocessing of banana wastes. Bioresour Technol 69:231–239

Krogh KBRM, Kastberg H, Jørgensen CI, Berlin A, Harris PV, Olsson L (2009) Cloning of a GH5 endoglucanase from genus *Penicillium* and its binding to different lignins. Enzyme Microb Technol 44:359–367

Kuhad RC, Singh A (1993) Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13:151–172

Kuhad RC, Singh A (2007) Lignocellulose biotechnology: future prospects. IK International Publication, New Delhi

Kuhad RC, Kumar M, Singh A (1994) A hypercellulolytic mutant of *Fusarium oxysporum*. Lett Appl Microbiol 19:397–400

Kuhad RC, Singh A, Eriksson K-EL (1997) Microorganisms and enzymes involved in the degradation of plant fibre cell walls. Adv Biochem Eng Biotechnol 57:45–125

Kuhad RC, Gupta R, Khasa YP (2010a) Bioethanol production from lignocellulosic biomass: an overview. In: Lal B (ed) Wealth from waste. Teri Press, New Delhi, pp 53–106

Kuhad RC, Gupta R, Khasa YP, Singh A (2010b) Bioethanol production from *Lantana camara* (red sage): pretreatment, saccharification and fermentation. Bioresour Technol 101:8348–8354

Kuhad RC, Mehta G, Gupta R, Sharma KK (2010c) Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by Saccharomyces cerevisiae. Biomass Bioenerg 34:1189–1194

Kuhad RC, Gupta R, Singh A (2011) Microbial cellulases and their industrial applications. Enzyme res 2011 (Article ID 280696):10p. doi:10.4061/2011/280696

Kung L Jr, Kreck EM, Tung RS, Hession AO, Sheperd AC, Cohen MA, Swain HE, Leedle JAZ (1997) Effects of a live yeast culture and enzymes on in vitro ruminal fermentation and milk production of dairy cows. J Dairy Sci 80:2045–2051

Lechner BE, Papinutti VL (2006) Production of lignocellulosic enzymes during growth and fruiting of the edible fungus *Lentinus tigrinus* on wheat straw. Process Biochem 41:594–598

Lee Y, Kim B, Lee B, Jo K, Lee N, Chung C, Lee Y, Lee J (2008) Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresour Technol 99:378–386

Leite RSR, Alves-Prado HF, Cabral H, Pagnocca FC, Gomes E, Da-Silva R (2008) Production and characteristics comparison of crude β-glucosidase produced by microorganism *Thermoascus aurantiacus e Aureobasidium pullulans* in agricultural wastes. Enzyme Microb Technol 43:391–395

Lewis GE, Hunt CW, Sanchez WK, Treacher R, Pritchard GT, Feng P (1996) Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. J Anim Sci 74:3020–3028

Li Y, Ding M, Wang J, Xu G, Zhao F (2006) A novel thermoacidophilic endoglucanase, Ba-EGA, from a new

cellulose-degrading bacterium, *Bacillus* sp. AC-1. Appl Microbiol Biotechnol 70:430–436

Li W, Zhang WW, Yang MM, Chen YL (2008) Cloning of the thermostable cellulase gene from newly isolated *Bacillus subtilis* and its expression in *Escherichia coli*. Mol Biotechnol 40:195–201

Li W, Huan X, Zhou Y, Qingyi M, Chen Y (2009) Simultaneous cloning and expression of two cellulase genes from *Bacillus subtilis* newly isolated from Golden Takin (*Budorcas taxicolor* Bedfordi). Biochem Biophys Res Commun 383:397–400

Liang Y, Feng Z, Yesuf J, Blackburn JW (2009) Optimization of growth medium and enzyme assay conditions for crude cellulases produced by a novel thermophilic and cellulolytic bacterium, *Anoxybacillus* sp. 527. Appl Biochem Biotechnol 160:1841–1852

Lima ALGD, Nascimento RPD, Bon EPDS, Coelho RRR (2005) *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. Enzyme Microb Technol 37:272–277

Liu Y, Zhang J, Liu Q, Zhang C, Qingsheng M (2004) Molecular cloning of novel cellulase genes *cel*9a and *cel*12a from *Bacillus licheniformis* GXN151 and synergism of their encoded polypeptides. Curr Microbiol 49:234–238

Liu RH, Dai SA, Chang FJ, Cheng WT, Shih YF (2009) Investigation on solubility of polymeric binder of xerographic toner and de-inking by emulsion process. J Taiwan Inst Chem Eng 40:84–90

Liu RH, Dai SA, Chang FJ, Cheng WT, Shih YF (2010) Highly efficient PET film-assisted adsorption process for the de-inking of xerographic wastepaper. J Taiwan Inst Chem Eng 41:344–351

Lo Y, Saratale GD, Chen W, Bai M, Chang J (2009) Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microb Technol 44:417–425

Lo Y, Lu W, Chen C, Chen W, Chang J (2010) Characterization and high-level production of xylanase from an indigenous cellulolytic bacterium *Acinetobacter junii* F6-02 from southern Taiwan soil. Biochem Eng J 53:77–84

Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE (1994) Purification, characterisation, and synergistic activity of a glucan-1,3-glucosidase and N-acetyl-b-glucosaminidase from *Trichoderma harzianum*. Phytopathology 84:398–405

Louime C, Abazinge M, Johnson E, Latinwo L, Ikediobi C, Clark A (2007) Molecular cloning and biochemical characterization of a family-9 endoglucanase with an unusual structure from the gliding bacteria *Cytophaga hutchinsonii*. Appl Biochem Biotechnol 136:127–137

Ludwig R, Haltrich D (2003) Optimisation of cellobiose dehydrogenase production by the fungus *Sclerotium (Athelia) rolfsii*. Appl Microbiol Biotechnol 61:32–39

Mahadevan SA, Wi SG, Lee DS, Bae HJ (2008) Site-directed mutagenesis and CBM engineering of Cel5A (*Thermotoga maritima*). FEMS Microbiol Lett 287:205–211

Maki M, Leung KT, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 5:500–516

Mekoo JLD, Yang Y, Li T, Sao R, Liu J (2010) Molecular cloning and expression of a synthetic gene encoding a β-glucosidase of *Aspergillus Niger* in the methylotrophic yeast *Pichia Pastoris*. Int J Biol 2:40–49

Mello-de-Sousa TM, Silva-Pereira I, Pocas-Fonseca MJ (2011) Carbon source and pH-dependent transcriptional regulation of cellulase genes of *Humicola grisea* var. *thermoidea* grown on sugarcane bagasse. Enzyme Microb Technol 48:19–26

Membrillo I, Sanchez C, Meneses M, Favela E, Loera O (2008) Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. Bioresour Technol 99:7842–7847

Michaud P, Belaich A, Courtois B, Courtois J (2002) Cloning, sequencing and overexpression of a Sinorhizobium meliloti M5N1CS carboxymethyl-cellulase gene. Appl Microbiol Biotechnol 58:767–771

Moon M, Anderson KL (2001) *Eubacterium cellulosolvens* alters its membrane protein, lipoprotein, and fatty acid composition in response to growth on cellulose. Anaerobe 7:227–236

Niranjane AP, Madhou P, Stevenson TW (2007) The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. Enzyme Microb Technol 40:1464–1468

Noe P, Chevalier J, Mora F, Comtat J (1986) Action of enzymes in chemical pulp fibres. Part II: enzymatic beating. J Wood Chem Technol 6:167–184

Ohara H, Karita S, Kimura T, Sakka K, Ohmiya K (2000) Characterization of cellulolytic complex (Cellulosome) from *Ruminococcus albus*. Biosci Biotechnol Biochem 64:254–260

Ozkose E, Akyo I, Kar B, Comlekcioglu U, Ekinci MS (2009) Expression of fungal cellulase gene in *Lactococcus lactis* to construct novel recombinant silage inoculants. Folia Microbiol 54:335–342

Pages S, Belaich A, Belaich JP, Morag E, Lamed R et al (1997) Species-specificity of the cohesin-dockerin interaction between *Clostridium thermocellum* and *Clostridium cellulolyticum*: prediction of specificity determinants of the dockerin domain. Proteins 29:517–527

Palackal N, Lyon CS, Zaidi S, Luginbuhl P, Dupree P, Goubet F, Macomber JL, Short JM, Hazlewood GP, Robertson DE, Steer BA (2007) A multifunctional hybrid glycosyl hydrolase discovered in an uncultured microbial consortium from ruminant gut. Appl Microbiol Biotechnol 74:113–124

Pang H, Zhang P, Duan CJ, Mo XC, Tang JL, Feng JX (2009) Identification of cellulase genes from the metagenomes of compost soils and functional characterization of one novel endoglucanase. Curr Microbiol 58:404–408

Pariza MW, Cook M (2010) Determining the safety of enzymes, used in animal feed. Regul Toxicol Pharmacol 56:332–334

Park CS, Kawaguchi T, Sumitani JC, Takada G, Izumori K, Arai M (2005) Cloning and sequencing of an

exoglucanase gene from *Streptomyces* sp. M23, and its expression in *Streptomyces lividans* TK-24. J Biosci Bioeng 99:434–436

Pazarlioglu NK, Sariisik M, Telefoncu A (2005) Treating denim fabrics with immobilized commercial cellulases. Process Biochem 40:767–771

Pere J, Paavilainen L, Siika-Aho M, Cheng Z, Viikari L (1996) Potential use of enzymes in drainage control of nonwood pulps. In: Proceedings of 3rd international non-wood fibre pulping and paper making conference, vol 2, Beijing, pp 421–30

Pommier JC, Fuentes JL, Goma G (1989) Using enzymes to improve the product quality in the recycled paper industry. Part 1: the basic laboratory work. Tappi J 72: 187–191

Pommier JC, Goma G, Fuentes JL, Rousset C, Jokinen O (1990) Using enzymes to improve the process and the product quality in the recycled paper industry. Part 2: industrial applications. Tappi J 73:197–202

Ponpium P, Ratanakhanokchai K, Kyu KL (2000) Isolation and properties of a cellulosome-type multienzyme complex of the thermophilic *Bacteroides* sp. strain P-1. Enzyme Microb Technol 26:459–465

Posta K, Wilson DB, Kukolya J, Hornok L, Beki E (2004) Cloning, characterization and phylogenetic relationships of *cel5B*, a new endoglucanase encoding gene from *Thermobifida fusca*. J Basic Microbiol 44:383–399

Pottkämper J, Barthen P, Ilmberger N, Schwaneberg U, Schenk A, Schulte M, Ignatiev N, Streit WR (2009) Applying metagenomics for the identification of bacterial cellulases that are stable in ionic liquids. Green Chem 11:957–965

Poutanen K (1997) Enzymes: an important tool in the improvement of the quality of cereal foods. Trends Food Sci Technol 8:300–306

Nishiyama Y, Sugiyama J, Chanzy H, Langan P (2003) Crystal structure and hydrogen bonding system in cellulose Ia from aynchrotron X-ray and neutron fiber diffraction. J Am Chem Soc 125(47): 14300–14306

Notley SM, Pettersson B, Wagberg L (2004) Direct measurement of attractive van der Waals' forces between regenerated cellulose surfaces in an aqueous environment. J Am Chem Soc 126(43):13930–13931

Rajoka MI, Malik KA (1997) Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. Biore sour Technol 59:21–27

Ramírez NR, García ERR, Calderón VC, Avitia CI, Valencia AT, Reyes MP (2008) Expression, characterization and synergistic interactions of *Myxobacter* Sp. AL-1 Cel9 and Cel48 Glycosyl Hydrolases. Int J Mol Sci 9:247–257

Rastogi G, Bhalla A, Adhikari A, Bischoff KM, Hughes SR, Christopher LP, Sani RK (2010) Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. Bioresour Technol 101:8798–8806

Rees HC, Grant S, Jones B, Grant WD, Heaphy S (2003) Detecting cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries. Extremophiles 7:415–421

Ribeiro O, Wiebe M, Ilmén M, Domingues L, Penttilä M (2010) Expression of Trichoderma reesei cellulases CBHI and EGI in *Ashbya gossypii*. Appl Microbiol Biotechnol 87:1437–1446

Rubini MR, Dillon AJP, Kyaw CM, Faria FP, Poças-Fonseca MJ, Silva-Pereira I (2009) Cloning, characterization and heterologous expression of the first *Penicillium echinulatum* cellulase gene. J Appl Microbiol 37:1365–2672

Sabathe F, Belaich A, Soucaille P (2002) Characterization of the cellulolytic complex (cellulosome) of *Clostri-dium acetobutylicum*. FEMS Microbiol Lett 217:15–22

Saha BC (2004) Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem 39:1871–1876

Scott RB, Piree SP, Lavigane J, Masri N, White CT, Thomasheck JJ (2010) Novel lignin resistant cellulase enzyme. US Patent 2010/0221778 A1

Shedova EN, Berezina OV, Lunina NA, Zverlov VV, Schwarz WH, Velikodvorskaya GA (2009) Cloning and characterization of a large metagenomic DNA fragment containing glycosyl-hydrolase genes. Mol Genet Microbiol Virol 24:12–16

Shima S, Kato J, Igarashi Y, Kodama T (1989) Cloning and expression of a *Clostridium cellobioparum* Cellulase Gene and its excretion from *Escherichia coli* JM109. J Ferment Bioeng 68:75–78

Sita NG (2010) Pure culture and metagenomic approaches to investigate cellulose and xylan degradation. MSc Thesis, University of Ontario Institute of Technology

Sreenath HK, Sudarshanakrishna KR, Santhanam K (1994) Improvement of juice recovery from pineapple pulp/residue using cellulases and pectinases. J Ferment Bioeng 78:486–488

Srinivasan K, Murakami M, Nakashimada Y, Nishio N (2001) Efficient production of cellulolytic and xylanolytic enzymes by the rumen anaerobic fungus, *Neocallimastix frontalis* in a repeated batch culture. J Biosci Bioeng 91:153–158

Sukumaran RK, Singhania RR, Pandey A (2005) Microbial cellulases—production, applications and challenges. J Sci Ind Res 64:832–844

Sun X, Liu Z, Zheng K, Song X, Qu Y (2008) The composition of basal and induced cellulase systems in *Penicillium decumbens* under induction or repression conditions. Enzyme Microb Technol 42:560–567

Tang B, Haibo P, Zhang Q, Ding L (2009) Cloning and expression of cellulase gene EG1 from *Rhizopus stolonifer* var. reflexus TP-02 in *Escherichia coli*. Bioresour Technol 100:6129–6132

Teeri TT, Koivula A, Linder M, Wohlfahrt G, Divne C, Jones TA (1998) *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? Biochem Soc Trans 26:173–178

Thirumale S, Rani DS, Nand K (2001) Control of cellulose formation by trehalose in Clostridium papyrosolvens CFR-703. Process Biochem 37:241–245

Toda H, Nagahata N, Amano Y, Nozaki K, Kanda T, Okazaki M, Shimozaka M (2008) Gene cloning of cellobiohydrolase II from the white rot fungus *Irpex lacteus* MC-2 and its expression in *Pichia pastoris*. Biosci Biotechnol Biochem 72:3142–3147

Tomme P, Warren RAJ, Gilkes NR (1995) Cellulose hydrolysis by bacteria and fungi. Adv Microb Physiol 37:1–81

Trivedi N, Gupta V, Kumar M, Kumari P, Reddy CRK, Jha B (2011) An alkali-halotolerant cellulase from *Bacillus flexus* isolated from green seaweed *Ulva lactuca*. Carbohydr Polym 83:891–897

Voget S, Leggewie C, Uesbeck A, Raasch C, Jaeger KE, Streit WR (2003) Prospecting for novel biocatalysts in a soil metagenome. Appl Environ Microbiol 69:6235–6242

Waeonukul R, Kyu KL, Sakka K, Ratanakhokchai K (2009) Isolation and characterization of a multienzyme complex (cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown on Avicel under aerobic conditions. J Biosci Bioeng 107:610–614

Wang WK, Kruss K, Wu JHD (1993) Cloning and dna sequence of the gene coding for *Clostridium thermocellum* Cellulase ss (cels), a major cellulosome component. J Bacteriol 175:1293–1302

Wang F, Li F, Chen G, Liu W (2009) Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. Microbiol Res 164:650–657

Ward OP, Singh A (2002) Bioethanol technology: developments and perspectives. Adv Appl Microbiol 51:53–80

Warnecke F, Luginbuhl P, Ivanova N et al (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450:560–565

Watanabe H, Tokuda G (2001) Animal cellulases. Cell Mol Life Sci 58:1167–1178

Wei H, Xu Q, Taylor LE II, Baker JO, Tucker MP, Ding S (2009) Natural paradigms of cell wall degradation. Curr Opin Biotechnol 20:330–338

Wen Z, Liao W, Chen S (2005) Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour Technol 96:491–499

Wilson DB, Irwin DC (1999) Genetics and properties of cellulases. Adv Biochem Eng Bio-Technol Recent Prog Bioconversion 65:1–21

Wulff AN, Carrer H, Pascholati SF (2006) Expression and purification of cellulase xf818 from Xylella fastidiosa in *Escherichia coli*. Curr Microbiol 53:198–203

Wyk JPH, Mogale MA, Seseng TA (2000) Saccharification of used paper with different cellulases. Biotechnol Lett 22:491–494

Xiao-Min W, Yu-Qi Qin, Yin-Bo Qu (2010) Molecular cloning and characterization of two major endoglucanases from *Penicillium decumbens*. J Microbiol Biotechnol 20(2):265–270

Zeng R, Xiong P, Wen J (2006) Characterization and gene cloning of a cold-active cellulase from a deep-sea psychrotrophic bacterium *Pseudoalteromonas* sp. DY3. Extremophiles 10:79–82

Zhang Y-HP, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: non-complexed cellulase systems. Biotechnol Bioeng 88:797–824

Zhang J, Zhong Y, Zhao X, Wang T (2010) Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced b-glucosidase and filter paper activity using strong artificial cellobiohydrolase 1 promoter. Bioresour Technol 101:9815–9818

Microbial Pectinases and Their Applications

7

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Abstract

In nature, microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the years. Among them, pectinolytic enzymes are of great significance and are one of the most important enzymes of the commercial sector. Microbial pectinases can be produced from bacteria, actinomycetes, yeasts and fungi. Pectinases are being used in several conventional industrial processes, such as fruit juice extraction and clarification, textile processing, tea and coffee fermentation, recovery of vegetable oils, retting and degumming of plant fibres and paper making. This chapter is aimed at reviewing the various types of pectinases and their biotechnological applications.

Keywords

Pectinase • Fruit juice extraction and clarification • Degumming • Retting • Pectinase genes

Introduction

Recent years have seen a great increase in industrial applications of pectinases owing to their significant biotechnological potential. Pectinases are a heterogenous group of related enzymes that hydrolyse the pectic substances present in the plant cell wall (Whitaker 1990). In nature, a wide variety of microorganisms, e.g. bacteria, yeasts, actinomycetes and filamentous fungi have been reported to produce pectinases (Kapoor et al. 2001; Hoondal et al. 2002; Kuhad et al. 2004; Jayani et al. 2005; Torres et al. 2006; Gupta et al. 2008; Murad and Azzaz 2011; Maleki et al. 2011). The pectinases have been used more commonly in the extraction and clarification of fruit juices and in wine production (Ribiero et al. 2010; Prathyusha and Suneetha 2011; Sandri et al. 2011), while they are also of great significance in other industrial applications as well, such as textile processing, degumming and retting of plant fibres, paper making and coffee and tea fermentations (Kashyap et al. 2001; Hoondal et al. 2002; Jayani et al. 2005; Aggarwal et al. 2008). At present there are several companies worldwide for producing

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Table 7.1 Some commercial pectinases

Supplier	Location	Brand name
C.H. Boehringer Sohn	Ingelheim, Germany	Panzym
Ciba-Geigy, A.G.	Basel, Switzerland	Ultrazyme
Kikkoman Shoyu, Co.	Tokyo, Japan	Sclase
Schweizerische Ferment, A.G.	Basel, Switzerland	Pectinex
Wallerstein, Co.	Des Plaines, USA	Klerzyme
Rohm, GmbH	Darmstadt, Germany	Pectinol, Rohament
Grinsteelvaeket	Aarthus, Denmark	Pectolase
Societe Rapidase, S.A	Seclin, France	Rapidase
Clarizyme Wallerstein, Co.	Des Plaines, USA	Klerzyme
Biocon Pvt. Ltd.	Bangalore, India	Pectinase
Novozyme	Denmark	Pectinex Mash
Lyven	France	Ly Peclyve PR
Danisco	Denmark	MaxLiq
AB Enzymes	Finland	Rohapect® MA Plus

Table 7.2 Composition of pectin in different fruits and vegetables (fresh weight)

Fruit	Pectic substance (%)	Fruit	Pectic substance (%)
Apple	0.5–1.6	Blackcurrant	0.8–1.1
Banana	0.7–1.2	Cranberry	0.8–1.2
Peaches	0.1–0.9	Grape	0.1–0.5
Strawberries	0.6–0.7	Lemon	1.8–2.2
Cherries	0.2–0.5	Carrot	1.2–1.5
Peas	0.9–1.4	Mango	0.3–0.5
Apricot	0.8–1.0	Pineapple	0.04–0.1
Oranges	0.5–3.5	Plum	0.7–0.9
Citrus peel	25–30	Raspberry	0.4–0.6
Blackberry	0.7–0.9		

commercial pectinases to be used in various industrial applications (Table 7.1).

Pectic Substances: Substrate of Pectinase

Pectic substances are glycosidic macromolecules of high molecular weight, which form the largest component of the middle lamella of higher plants (Alkorta et al. 1998; Kashyap et al. 2001; Jayani et al. 2005; Almeida et al. 2005; Pedrolli et al. 2009), accounting approximately 0.1–3.0% of the fresh weight of plants (Table 7.2). Chemically, they are a complex colloidal acidic polysaccharides composed of galacturonic acid residues linked by α-1,4-glycosidic bonds (Fig. 7.1), partially esterified by methyl ester and partially or completely neutralised by one or more basic ions such as sodium, potassium or ammonium (Limberg et al. 2000; Kuhad et al. 2004).

American Chemical Society classified pectic substances into four main types as follows (Alkorta et al. 1998):

Protopectin: It is the water-insoluble pectic substance present in intact plant tissue. Protopectin on restricted hydrolysis yields pectin or pectic acids.

Pectic acid: It is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.

Pectinic acids: It is the polygalacturonan chain that contains up to 75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates.

Pectin (*polymethylgalacturonate*): It is polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall.

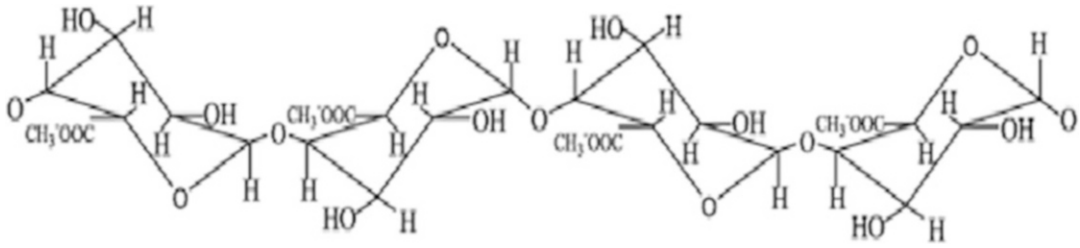

Fig. 7.1 Primary structure of pectic substances (Rexova-Benkova and Markovic 1976)

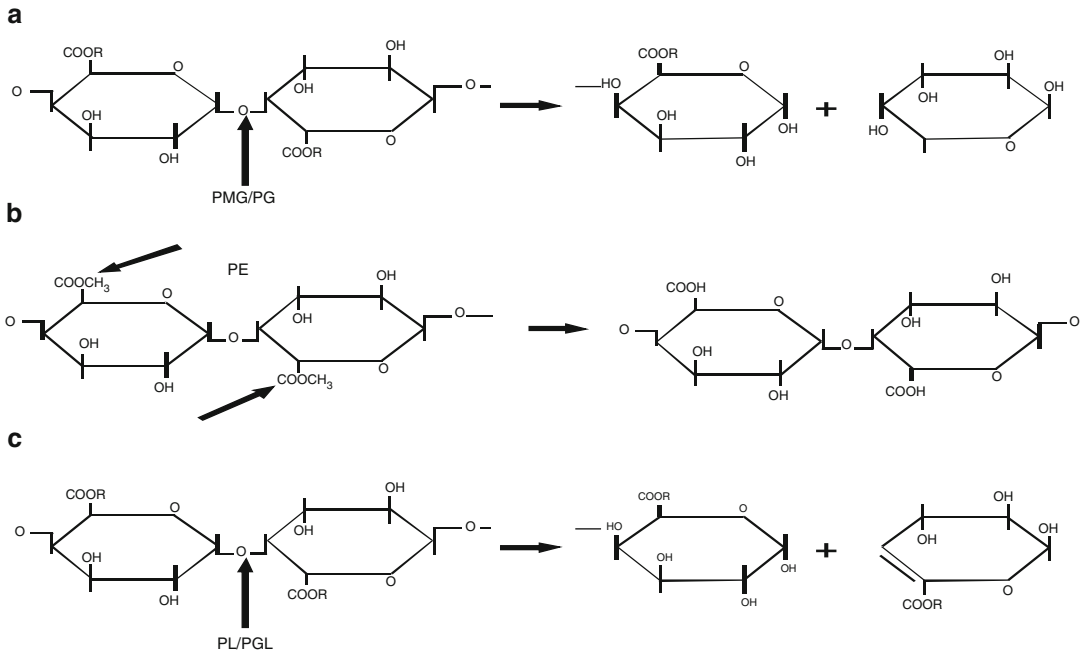

Fig. 7.2 Different types of pectinases and their mode of action on pectic substances (**a**) R=H for PG and CH_3 for PMGL (**b**) PE and (**c**) R=H for PGL and CH_3 for PL. The *arrow* indicates the place where the pectinases reacts with pectic substances. *PMGl* polymethylgalacturonate lyase, *PG* polygalacturonase, *PE* pectinesterase, *PL* pectin lyase (Pedrolli et al. 2009)

Pectinolytic Enzymes

Pectic substances are ubiquitous in the plant kingdom, and their efficient utilisation could enhance the economic competitiveness of bioconversion processes intended to compete with conventional industrial processes (Kapoor et al. 2001; Kapoor and Kuhad 2002). Therefore, nowadays a significant interest in the degradation of pectic substances with pectinases has been generated. Pectinases based on their mode of action (Fig. 7.2) can be divided into three broad groups:

(a) *Protopectinases*: Protopectinases or pectinosinases (PPase) are the enzymes that catalyse the solubilisation of protopectin to produce highly polymerised soluble pectin (Brinton et al. 1927). On the basis of their reaction mechanism, protopectinases are classified into two types, i.e. A type and B type. A type protopectinase reacts with the inner site, i.e. the polygalacturonic acid region of protopectin, whereas the B type protopectinase reacts on the outer site, i.e. on the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents (Sakai et al. 1993).

(b) *Depolymerising enzymes* which break α-1, 4, linkages in the principal pectin chain such as:
   (i) *Polygalacturonase*: Polygalacturonases (PGases) are the pectinolytic enzymes that catalyse the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge. The PGases involved in the hydrolysis of pectic substances are of two types, i.e. endo-polygalacturonases and exo-polygalacturonases. Endo-PG also known as poly(1,4-α-D-galacturonide) glycanohydrolase catalyses random hydrolysis of α–1,4-glycosidic linkages in the pectic acid, while exo-PG also known as poly(1,4-α-D-galacturonide) galacturonohydrolases catalyses hydrolysis in a sequential fashion of α-1,4-lycosidic linkages on pectic acid (Kashyap et al. 2001).
   (ii) *Polymethylgalacturonases*: Polymethylgalacturonases (PMGL) catalyses the hydrolytic cleavage of α-1,4-glycosidic bonds in pectin backbone, preferentially highly esterified pectin-forming 6-methyl-D-galacturonate (Jayani et al. 2005; Pedrolli et al. 2009).
   (iii) *Lyases*: Lyases or transeliminases are the enzymes that perform non-hydrolytic breakdown of pectates or pectinates by a transeliminative split of the pectic polymer (Sakai et al. 1993; Kashyap et al. 2001; Jayani et al. 2005; Pedrolli et al. 2009). They are classified as endo-pectate transeliminase (pectate lyase, PGL) and endo-pectin transeliminase (pectin lyase, PL). Pectate lyase cleaves glycosidic linkages preferentially on polygalacturonic acid forming unsaturated product through transelimination reaction. Pectate lyases are classified as endo-PGL that acts towards substrate in a random way and exo-PGL that catalyse the substrate cleavage from nonreducing end. Pectin lyase (PL) catalyses the random cleavage of pectin, preferentially high-esterified pectin, producing unsaturated methyloligogalacturonates through transelimination of glycosidic linkages

(c) *Demethoxylating* enzymes such as *pectinesterase*: Pectinesterase (PE) often referred to as pectin methylesterase, pectase, pectin methoxylase, pectin demethoxylase and pectolipase is a carboxylic acid esterase and belongs to the hydrolase group of enzymes (Whitaker 1990). It catalyses the de-esterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Cosgrove 1997; Kashyap et al. 2001; Malvessi and Silveira 2004; Jayani et al. 2005; Pedrolli et al. 2009).

The action of polygalacturonase, pectin methyl esterase and pectin lyase leads to extensive degradation of middle lamella and cell wall pectin, and this property of pectinases makes them useful in various industrial applications (Ribiero et al. 2010).

Properties of Pectinases

During the last few decades, pectinases from various microorganisms have been studied extensively for their use in various industries. Various properties of pectinases, viz. molecular weight, pH and temperature optima and stability have been summarised in Table 7.3.

Applications of Pectinases

Acidic pectinases are mainly used for the extraction and clarification of fruit juices and wines. Alkaline pectinases are employed for the processing and degumming of plant fibres, in paper making, etc. (Gupta et al. 2008). Some of the industrial applications of pectinases have been discussed as follows:

Fruit Juice Industries

The juices from a wide variety of fruits such as apple, pear, plum, mango, banana, grape, apricot, orange, guava, papaya, strawberry, raspberry and blackberry can be extracted to produce natural beverages. However, the most important

Table 7.3 Characterisation of some microbial pectinases

Pectinase producer	Nature	Molecular weight (kDa)	pI	Optimum temperature (°C)	Optimum pH	References
P. italicum	PMGL	22	8.6	40	6.0–7.0	Alana et al. (1990)
B. macerans	PGL	35	10.3	60	9.0	Miyazaki (1991)
A. japonicas	PMGL	–	7.7	55	6.0	Dinnella et al. (1994)
A. carbonarius	Endo-PG	61 (PG I)	–	55	4.0	Devi and Rao (1996)
		42 (PG II)	–	50	4.1	
		47 (PG III)	–	55	4.3	
Pythium splendens	PMGL	23	8.0	–	8.0	Chen et al. (1998)
Bacillus sp.	PGL	38	–	69	11.0	Singh et al. (1999)
Bacillus sp. KSM-P7	PGL(Pel 7)	33	10.5	60–65	10.5	Kobayashi et al. (1999)
S. cerevisiae	Endo-PG	39	–	45	5.5	Ganivors et al. (2000)
Bacillus sp. DT7	PL	106	–	60	8.0	Kashyap et al. (2000)
A. japonicas	PE	46(PE I)	3.8	–	4.0–5.5	Hasunuma et al. (2003)
		47(PE II)	3.8	–	4.0–5.5	
A. japonicas	Endo-PG	38 (PG I)	5.6	30	4.0–5.5	Semenova et al. (2003)
	Endo-PG	38 (PG II)	3.3	30	4.0–5.5	
A. Japonicas	PL	50	3.8	40–50	4.5–5.5	Semenova et al. (2003)
A. japonicas	Pectin esterase I	46	3.8	40–50	4.5–5.5	Semenova et al. (2003)
	Pectin esterase II	47	3.8	40–50	4.5–5.5	
Mucor flavus	Endo-PGL	40	8.3	45	3.5–5.5	Gadre et al. (2003)
F. moniliforme	PGL	–	–	–	8.5	Dixit et al. (2004)
A. kawachii	Endo-PG	60	–	37	4.0	Esquvel and Voget (2004)
R. oryzae	Endo-PG	31	–	45	4.5	Saito et al. (2004)
B. macerans	PGL	–	–	63–67	8.0–8.5	Morozova et al. (2006)
B. pumilus	PGL	37.3	8.5	70	8.5	Klug-Santner et al. (2006)
B. subtilis	PGL(Pel C)	42	7.8	65	10.0	Soriano et al. (2006)
A. flavus		–	–	50	8.0	Yadav et al. (2008)
Streptomyces lydicus	Exo-PG	43		50	6	Jacob et al. (2008)
A. giganteus	Exo-PG	–	–	55	6.0	Pedrolli et al. (2008)
A. giganteus	PL	–	–	50	8.5	Pedrolli et al. (2008)
P. citrinum	PL	31	–	50	6.5	Rasheedha Banu et al. (2010)
B. pumilus	PL	25	–	60	6	Nadaroglu et al. (2010)
B. subtilis RCK	Exo-PG	54	9.1	60	10.5	Gupta et al. (2008)
A. niger URM4645	Endo-PG	–	–	50	5	Maciel et al. (2011)
	PL			50	5	
	Exo-PG			40	7	

characteristic affecting the extraction of juice is the fruit cell wall, which is a complex structure of interwoven polymers composed of bundles of crystalline cellulose microfibres embedded in an aqueous gel of hemicellulose and pectin (Alkorta et al. 1998).

As a result, application of pectinases, cellulases and hemicellulases (collectively called macerating enzymes) has been observed useful in the extraction and clarification of fruit and vegetable juices (Kaur and Satyanarayana 2004; Unejo and Pastore 2007; Ribiero et al. 2010; Prathyusha and Suneetha 2011). Pectinases degrade pectin and other high molecular weight components in the cell wall, resulting in increased juice yield and decreased viscosity, thus giving a crystalline appearance to the final product with reduction in filtration time up to 50%

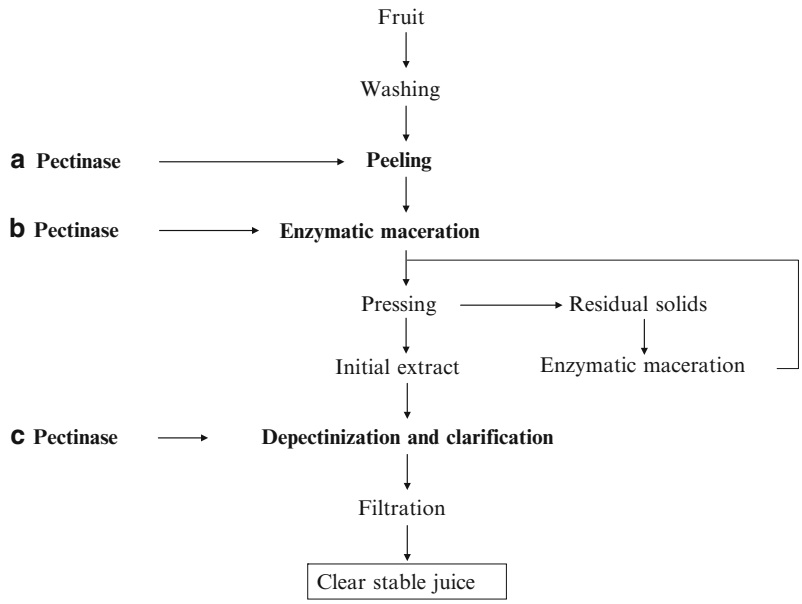

Fig. 7.3 Figure illustrating various steps in enzymatic processing of fruit juice

(Sandri et al. 2011; Maleki et al. 2011). Figure 7.3 depicts various steps of fruit juice processing with pectinases, which are as follows:

A. Peeling

Peeling is the first step in the processing of fruits and vegetables for juice extraction. Conventional methods of peeling (chemical, mechanical, steam and freeze peeling) cause high peeling losses and damage the flesh, affecting the quality of the fruit. Enzymatic peeling is thus suggested a more recent alternative (Pagan et al. 2006). The main advantages of enzymatic peeling are good quality of the final product, as well as reduced heat treatment and industrial waste.

It is an advantageous fact that the process does not require extensively harsh treatments (high temperatures), and as a result, the peeled fruits retain their structural integrity and fresh fruit properties. Pectin, cellulose and hemicellulose are the polysaccharides responsible for the adherence of the peel to the fruit. Therefore, treating the fruit with the corresponding enzymes provides the peeling of the fruit. Toker and Bayindirli (2003) used commercial enzymes (concentrated enzyme with pectinase, hemicellulase and cellulase activities) for enzymatic peeling of apricots, nectarines and peaches (stone fruits) and observed that enzymatic peeling was successful at moderately high temperatures concluding that the enzymatic peeling could thus be an alternative to mechanical or chemical peeling of stone fruits. Similarly, Pagan and co-workers (2006) optimised the conditions of temperature and concentration of the enzymatic preparation (polygalacturonases, hemicellulases and arabinases) for peeling of oranges and concluded that by treating the fruit with pectinase concentration of 5.0 ml/30 g peel for 28 min at 40.4°C produced the maximum weight loss, indicating a good peeling efficiency. In yet another report, Pretel and group (2007a, b) observed that peeling of different varieties of orange can be achieved with pectinases at 40°C.

B. Enzymatic Maceration

In the process of extracting juice, pectinases can be used to obtain a higher yield of sugar and soluble solids, resulting in a higher juice yield (Ribiero et al. 2010). In the production of white grape and red grape juice, pectinases have an important role in depectinisation to increase juice yield with the highest extraction of the

pigment that is naturally present in the grape. In general, more than 95% of the soluble solids from the fruit can be extracted by enzymatic treatment that would otherwise cause haze. By reducing fruit mash viscosity and improving solid/liquid separation, pectinases also increase colour extraction and juice volume (Rashmi et al. 2008). Moreover, the enzymatic maceration also results in subtle but generally beneficial changes in fruit juice flavour. Demir and co-workers (2001) observed that pectinase treatment of carrot puree decreased its viscosity from 90 to 6.5 poise, while the dry matter content and the total yield were found to be increased due to polysaccharide degradation. In the production of white grape and red grape juice, pectinases have an important role in depectinisation to increase juice yield. Significant improvements were obtained in the extraction, colour intensity and stability of wine treated with the recombinant yeast strains expressing pectate lyase and polygalacturonase genes by Louw and group (2006). Dzogbefia and Djokoto (2006) treated 200 g of papaya mash with different doses of the pectic enzyme extract and observed a rapid increase in flow rate of the free-run juice. Interestingly, Oliviera and co-authors (2006) observed that apple juice processing by enzymatic liquefaction yielded 83.5% sugars with 16.5% of pomace, while processing by pressing resulted in a yield of 64.5% sugars with 35.5% of pomace, thereby demonstrating that the enzymatic liquefaction process presents practical advantage over the process of extraction by pressing by minimising the production of pomace, the main factor that accounts for the high water retention in the conventional extraction systems, thereby providing a clean technology for apple juice processing. In another report, Lieu and Le (2010) observed that enzymatic treatment after sonication treatment of grape mash increased extraction yield by 7.3% and shortened the treatment time four times. Similarly, there are also several reports on the enhanced recovery and clarification of juices from dates, peach, plum, apricot and pineapple (Abbes et al. 2011; Joshi et al. 2011; Tran and Lee 2011).

C. Clarification

One of the major problems encountered in the preparation of fruit juices and wine is the cloudiness which is primarily due to the presence of pectins. Several authors have successfully used pectinolytic enzymes for clarification of fruit juices and wine. Singh and Gupta (2003) clarified apple juice using pectinolytic enzyme and gelatin. Lee and co-workers (2006) optimised conditions for enzymatic clarification of banana juice and achieved better clarity (decreased viscosity and turbidity) in the enzyme-treated juice. Sin and group (2006) used pectinase enzyme for clarification of sapodilla juice and recommended 0.1% enzyme concentration at 40°C for 120 min optimum for efficient clarification. In another report, Rai and co-workers (2007) studied the effect of various pretreatment methods on the clarification of mosambi juice and observed that maximum permeate flux during ultrafiltration was observed with enzymatic treatment followed by adsorption using bentonite. Similarly, Kareem and Adebowale (2007) clarified orange juice using crude fungal pectinase and obtained 51% reduction in the viscosity of the treated juice with a yield of 97% compared to the 73% yield in the untreated juice. Liew Abdullah and group (2007) established the optimum conditions for the enzymatic treatment of carambola fruit juice and reported that 0.1% enzyme concentration at 30°C for 20 min gave the highest clarity of the juice compared to the control. Cheirslip and Umsakul (2008) achieved fourfold higher clarity in the enzyme-treated banana wine compared with the control. Pinelo and co-authors (2010) concluded that pectin contributes to turbidity development during cold storage of cherry juice. Thus, the use of pectinases will help in increasing clarity of cherry juice. Vijayanand and group (2010) optimised clarification of litchi pulp with different doses of pectinase. The enzyme facilitated removal of insoluble solids and increased juice extraction. Nakkeeran and co-authors (2011) used polygalacturonase produced from *Aspergillus carbonarius* for the extraction and clarification of apple juice and achieved better yields and clarity (decreased viscosity) in enzymatically treated juice than the untreated juice. While, Yuan and co-workers

(2011) showed that the addition of endo-PG 1 from *Penicillium* sp. CGMCC 1669 and a commercial pectate lyase increased the efficiency of juice clarification reducing the intrinsic viscosity of apple juice by 33.1% (Yuan et al. 2011). In another report, Diaz and group (2011) used exo-polygalacturonase for clarification of grape must and achieved decrease in turbidity by 97.5%. A significant improvement in colour and clarity scores of plum, peach, pear and apricot juices was achieved using pectinase from *A. niger* (Joshi et al. 2011). Sandri and co-workers (2011) used fungal pectinases for clarification of apple, butia palm fruit, blueberry and grape juices and obtained greater clarification in enzyme-treated juice than in untreated juice.

Degumming of Plant Fibre

The removal of heavily coated, noncellulosic gummy material from the cellulosic part of plant fibres is called degumming and is necessary prior to the utilisation of fibres for textile making (Said et al. 1991). In a classical degumming process, this gum is removed by treatment of decorticated fibres with hot alkaline solution with or without application of pressure (Cao et al. 1992). In addition to the high consumption of energy, this process also results in serious environmental pollution. Biotechnological degumming using pectinases presents an ecofriendly and economic alternative to the conventional chemical process (Jayani et al. 2005). The use of pectinases in industrial processes is usually linked to a reduced consumption of energy as well as chemicals and thus beneficial for the environment (Demarche et al. 2011). Several authors have successfully used pectinolytic enzymes for degumming of plant fibres. Sharma and Satyanarayana (2006) applied pectinase produced from *B. pumilus* dcsr1 for treatment of ramie fibres and observed that the enzyme selectively degraded only the noncellulosic gummy material of the fibre, making the enzyme choice for degumming process. Jacob and co-workers (2008) treated handstripped sun-dried fibres with crude pectinase obtained from *Streptomyces lydicus* and observed that the fibre cells were intact in the control, while the cells were separated in the enzyme-treated sample when observed under scanning electron microscope. Sharma and group (2011) used pectinase from *Pseudozyma* sp. for degumming of flax fibres and achieved 4471 ± 19.5 μg g^{-1} dry fibre galacturonic acid with maximum weight loss of 11 ± 1.2% after 12 h of incubation.

A combined microbial and enzymatic process can also be used to reduce the consumption of energy and chemicals and achieve a better degumming (Deshpande and Gurucharanam 1985). Kapoor and co-workers (2001) observed that neither of the two treatments (chemical and enzymatic) alone is sufficient for an efficient degumming process. When chemically treated fibres were subsequently treated with polygalacturonase from *Bacillus* sp. MG-cp-2, a complete removal of gummy material was observed in ramie and sunn hemp fibre. The reason to this effect may be that when the fibre was treated with chemical, it would have caused greater porosity, softness, swelling and separation of the fibre, thus rendering it more accessible to enzymatic attack. Similarly, Kashyap and co-authors (2001) used combined (chemical and enzymatic) treatment to degum buel bast fibres and observed that the chemically treated buel fibres when subsequently treated with optimised (400 U/ml) doses of crude pectinase resulted in the release of maximum amount of galacturonic acid (575 μmol/g dry fibres) and a decrease in dry weight (43%) of the fibres. Basu and group (2009) also used combined degumming (enzymatic and chemical) process with *B. pumilus* strain DKS1 to degum ramie bast fibres on a large scale (400 kg) and achieved more than 20.81% increase of single fibre tenacity.

Retting of Plant Fibres

Retting is a fermentation process in which certain bacteria and fungi decompose the pectin of the bark and release fibre to be used for linen making in textile industry. Commercially, retting is done by one of the two basic forms (anaerobic retting

and dew retting). Anaerobic retting or water retting is achieved by submerging straw sheaves in water pits, in concrete tanks or in running freshwater. However, the process produces environmentally unacceptable fermentation waste (Sharma and Van Sumere 1992) and was therefore discontinued in western countries. While in dew retting (an aerobic process), plant straw is thinly spread on the ground and exposed to the action of the fungi and aerobic bacteria for 2–10 weeks. However, there are also several major disadvantages of dew retting such as dependence on geographical regions, coarser and lower-quality fibre, less consistency in fibre characteristics and occupation of agricultural fields for several weeks (Van Sumere 1992). Dew retting further results in a heavily contaminated fibre that is particularly disadvantageous in cotton textile mills. Enzyme retting was thus evaluated as a replacement for traditional microbial retting methods. Enzymatic retting is faster, readily controlled and produces fewer odours. Van Sumere and Sharma (1991) evaluated Flaxzyme, a commercial enzyme mixture from Novo Nordisk (Denmark) which consists of pectinases, hemicellulases and cellulases, at a concentration of 3 g/l for enzyme retting and produced fibre fineness, strength, colour and waxiness comparable to the best water-retted fibre. Akin and co-workers (2001) used Viscozyme L, a pectinase-rich commercial enzyme product, and ethylenediaminetetraacetic acid (EDTA) for treating Ariane fibre flax and North Dakota seed flax straw residue and produced the best test yarns. Evans and group (2002) compared enzyme retting of flax fibres with the water-retted fibre and achieved 62% increase in enzyme-retted fibre yield, while fibre strength did not differ between the two treatments. Akin and co-authors (2007) optimised enzyme retting of flax with pectate lyase and observed that enzymatic treatment at for 1 h at 55°C followed by treatment with EDTA for 24 h provided the best fibres. Saleem and co-workers (2008) observed that treating bast fibres with pectinases also improves mechanical characteristics of reinforced polypropylene in terms of decreased tensile strength and reduced cross-sectional area. Alix et al. (2012) compared mechanical properties of pectate lyase treated green flax fibres with dew-retted fibres and observed improvement in the properties of enzyme-treated fibres. Thus, apart from being environment-friendly, enzymatic method also improves the properties of the retted fibre.

Bioscouring of Cotton Fibres

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and ecofriendly manner, replacing toxic caustic soda used for the purpose earlier (Hoondal et al. 2002; Sawada and Ueda 2001). An additional asset of this process is that besides being energy conservative and more environmental friendly, pectinolytic enzymes do not affect the cellulose backbone, thus drastically limiting fibre damage. Klug-Santner and co-workers (2006) reported up to 80% of pectin removal from the outer layer of cotton by a purified endo-pectate lyase from *B. pumilus* BK2. Wang and co-authors (2007) optimised bioscouring condition of cotton-knitted fabrics with an alkaline pectinase from *B. subtilis* WSHB04-02 by using response surface methodology and achieved a desired pectin removal percentage companied with adequate wettability. In another report, Aggarwal and group (2008) employed cutinase and pectinase for cotton bioscouring and reported that the treatment is effective in the degradation of cotton waxes and pectin, allowing the design and introduction of a competitive innovative enzymatic scouring process. Hebeish and co-workers (2009) investigated conditions for effective bioscouring of cotton-based fabrics and observed that the bioscoured substrates exhibit fabrics performances comparable to the conventional alkali scouring. Recently, Abdel-Halim and co-authors (2010) carried out bioscouring of cotton fabrics with combinations of different pectinase preparations and different surfactants followed by emulsification posttreatment and achieved higher hydrophilicity along with smooth and clear fibre surface in the treated fibre when visualised under scanning electron microscopy.

Paper Making

Pectinases depolymerise polymers of galacturonic acids, subsequently reducing the cationic demand of pectin solutions and the filtrate resulting from the peroxide bleaching, thereby solving retention problems in pulp bleaching (Reid and Ricard 2000; Viikari et al. 2001). During paper making, it has also been found that bleached pulp contains a substantial amount of undesired pectins. By incorporating pectinase in the bleached or alkali-treated pulp, such harmful pectins in the aqueous phase of the pulp are degraded. Ahlawat and co-workers (2007) investigated the suitability of pectinase produced from *B. subtilis* SS in pulp bleaching for paper making and showed an increase in brightness (4.3%), whiteness (14.8%) and florescence (65.3%) and reduction in kappa number (15%), permanganate number (6.1%) and chemical oxygen demand in the treated pulp. In another study, the enzymatic prebleaching of kraft pulp with xylano-pectinolytic enzymes from the same bacterial isolate resulted in 25% reduction in active chlorine consumption in subsequent bleaching stages without any decrease in brightness along with improvement in pulp properties (Kaur et al. 2010).

Extraction of Vegetable Oils

Canola, coconut, sunflower seed, palm and olive oil are traditionally produced by extraction with organic solvents, most commonly hexane that is a potential carcinogen. Addition of cell wall degrading enzymes also improves the oil quality in terms of increased phenolic compounds with high antioxidant activities (Vierhuis et al. 2001). Cell wall degrading ability of pectinolytic enzymes also allows their use for vegetable oil extraction in aqueous process (Kashyap et al. 2001). The mild conditions during the process ensure the stability of the extracted components, resulting in better oil quality in terms of oxidative stability parameters. As the process takes place in water, degumming of the extracted oil is unnecessary since the phospholipids are retained in the residual solid phase (Latif et al. 2008). Moreau and co-workers (2004) achieved corn oil yields of about 35% using commercial pectinase as against 27% in hexane-extracted sample, while the chemical composition of oil obtained by both the treatments was similar. Latif and group (2008) concluded that an aqueous enzyme-assisted extraction process for canola oil has the potential to be an environment-friendly alternative to solvent extraction and has the added benefit that it can simultaneously recover high-quality protein for human consumption.

Functional Foods

Pectin and pectic polysaccharides are emerging as bioactive food ingredients. Grapefruit pectin used industrially as a stabiliser and as a supplement to baby food improves nutrition and physical development of children. These oligogalacturonides and their breakdown products by pectinolytic enzymes are classified as 'probiotics' because they are not hydrolysed in the upper gastrointestinal tract and can be used as health promoters in human and animal nutrition, stimulating selective growth of species of resident bacteria in the intestinal lap (Lang and Dornenburg 2000).

Coffee and Tea Fermentation

Fermentation of tea and coffee with pectinases accelerates the fermentation process. Enzymatic treatment removes mucilagenous coat from coffee beans and destroys the foam-forming property of instant tea powders by destroying pectins, thus improving the quality of the final product (Jayani et al. 2005; Pedrolli et al. 2009). According to Angayarkanni and co-workers (2002), adding pectinases in association with cellulases and hemicellulases to the tea-leaf fermenting bath raises the tea quality index by 5%. Masaud and Jespersen (2006) concluded that pectinolytic enzymes of yeast are involved in the degradation of pectin during coffee fermentation.

Improvement in the Extraction of Cassava Starch

Cassava pulp, the solid residue produced after extraction of starch, contains a significant proportion of starch granules (68%, dry basis) and fibre (27%, dry basis). The high fibre content probably reduces the extraction of remaining starch keeping the beads together and stuck in a fibrous network. This network can be disrupted by enzymatic methods, based on the application of a mixture of pectinases and cellulases that destroy the structural integrity of the matrix responsible for trapping of beads, exposing and releasing the starch (Sriroth et al. 2000).

Improvement in Antioxidant Property of Juices

Treatment with pectinases also increase the phenolic and antioxidant content of fruit juices whose potential benefits for human health have been recognised in recent years. The addition of commercial pectolytic enzymes is a common practice in winemaking to increase phenolic content of wines, especially anthocyanins. These enzymes may also improve the stability, taste and structure of red wines, because not only anthocyanins are released from the skins but also tannins bound to cell walls may be extracted because of enzymatic action. Landbo and Meyer (2004) reported that the yield, anthocyanin level, level of total phenolics as well as clarity of black currant juice were improved by using pectinolytic enzymes. Similarly, Kelebek and co-workers (2007) observed that the wines produced by pectinase treatment were higher in total phenolics, tannins and colour intensity and concluded that the increase in colour intensity may be due to an increase in polymeric anthocyanin content and/or due to co-pigmentation effects caused by the enhanced extraction of other phenolic fractions by pectinase treatment. In another report, Markowski and co-authors (2009) applied two commercial pectinolytic enzymes in apple juice processing and found an increase in the content of phenolic compounds. Armada and co-workers (2010) observed that the wines obtained from the enzymatic maceration showed highest content of varietal compounds and other desirable compounds such as ethyl esters or phenylethyl acetate.

Cloning of Pectinase Genes

In the light of economic benefits and biotechnological applications of pectinolytic enzymes, screening and designing of new pectinases with higher efficiency and specificity via genetic manipulations has become a major focus of researchers. Use of recombinant DNA technology facilitates the economic production of large quantities of pure pectinases and the engineering of tailor-made enzymes for specific applications. Many microbial pectinase genes have been cloned over the past years and still the research is focused in this area. A few of them have been listed in Table 7.4.

The advantages of use of recombinant DNA technology in pectinase production are listed as follows:

(a) *Increased Yield of Enzyme*: There are several reports where mutagenesis and protoplast fusion had shown significant improvement in the yield of pectinase production. Cao and co-workers (1992) identified two mutants of alkalophilic *Bacillus* sp. NTT33 resistant to catabolic repression, producing polygalacturonase activities up to 82.4% higher than wild type. Po16, a *P. occitanis* mutant presented improved cellulase and pectinase production as compared to wild type strain (Jain et al. 1990). A prototrophic hybrid was developed after protoplast fusion of auxotrophic mutants of *Aspergillus* sp. CH-Y-1043 and *A. flaviceps* ATCC 16795, which showed 15-fold increase in endo-PGase production than the wild strain of *Aspergillus* sp. Similarly, interspecific protoplast fusion of *A. niger* and *A. carbonaria* resulted in hybrids with higher production of both endo- and exo-pectinases (sixfold higher than the wild type strain) and improved growth on SSF with wheat bran as a sole source of nutrients (Kavitha and Kumar 2000). Antier and group (1993) isolated pectinase

Table 7.4 Cloning and expression of pectinase from various microorganisms

Parent strain	Gene cloned	Host for cloning	Vector for cloning	Vector for expression	Reference
A. oryzae KBN616	*Pg*	*Escherichia coli* MV1184	pUC18	–	Kitamoto et al. (1993)
A. aculeatus	*Rhg A*	*E. coli* BB4	pBluescript (SK+)	λEMBL4	Suykerbuyk et al. (1995)
A. flavus	*Pec A, Pec B*	*E. coli* LE392	EMBL3	pCFC80	Whitehead et al. (1995)
P. janthinellum	*Pga*	–	pSTA14	pSRI-019	Ishida et al. (1997)
Pseudomonas syringae pv. Lachrymus 859	*Pel s*	*P. syringae* BUVS 1	pCPP34 pCPP$&	–	Bauer and Collmer (1997)
A. niger	*PgaE*	–	pUC18, pPROM-H	pGW635	Parenicova et al. (1998)
S. cerevisiae IM1-8b	*Pgu1*	*E. coli*	pBluescript (SK+)	pBEJ16, pYES2	Blanco et al. (1998)
Botrytis cinerea	*Endo-PG*	*E. coli* DH5α	pBluescript (SK/KS)	λEMBL3	Wubben et al. (1999)
A. tubingensis	*XghA*	–	–	pCVlacK	Vlugt-Bergmans et al. (2000)
S. cerevisiae	*Pgl*	–	–	–	Ganivors et al. (2000)
A. niger	*Pg*	*E. coli* DH5α	–	pGW635	Pagès et al. (2000)
Bacillus sp. *Strain KSM-p15*	*Pectate lyase*	*E. coli* HB101	pHSG398	–	Ogawa et al. (2000)
Bacillus sp.	*PehK*	–	pHV300PLK	pHYPEHK	Sawada et al. (2001)
Fusarium circinatum	*Fcpg*	–	PGEMT	–	Chimwamurombe et al. (2001)
Bacterium strain ANT/505	*Pectate lyase*	*E. coli* BL21	pUC 18	pRSET – A	Truong et al. (2001)
Geotrichum candidum Ap2	*Ap2pg1*	*E. coli* DH5α	pBluescript (SK+)	–	Nakamura et al. (2002)
Treponema pectinovorum	*PelA*	*E. coli* DH5α	pBluescript II (SK+)	pQE30	Walker and Ryan (2003)
Bacillus alcalophilus NTT 33	*Pectate lyase*	*E. coli*	pBluescript ks(t) II		Zhai et al. (2003)
B. subtilis 168	*YuvA*	*E. coli* 5 K	pJF1184E	pET 28a	Soriano et al. (2006)
P. occitanis	*PnaI*	*E. coli* TOP1OF	pUC II	–	Lahiani et al. (2008)
A nidulans	*Pectate lyase*	*E. coli*	pMD18-T	pet 28(a)	Zhao et al. (2007)
B. subtilis	*Pectate lyase*	*E. coli*	pPIC9K	pPIC9K	Zhuge et al. 2008
P.griscoroseum	*PG*	*E. coli*	–	pAN52pgg2	Teixeira et al. (2011)

hyperproducing UV mutants of *A. niger* by looking for a deoxyglucose resistance phenotype. Sieiro and co-workers (2003) reported that the recombinant endopolygalacturonase produced by heterologous expression of the *Saccharomyces* PGU1 gene in *Schizosaccharomyces pombe* had higher Km value, thermostability and pH stability than native enzyme and was more efficient in reducing the viscosity of polygalacturonic acid.

(b) *Production of Pure Enzymes*: Large varieties of industrial applications of the pectinases require action of a single pectic enzyme of high purity or that of several enzymes in the appropriate proportions (Ceci and Lozano 1998). Accordingly, it would be of considerable use to produce the different pectic enzyme individually, and in large quantities, so that they can be mixed as required. Pure pectin lyases are suitable for reducing viscosity of cloud stable fruit juices rather than conventional enzymes which contain interfering enzyme activities such as pectinesterase, which leads to production of methanol. Using the pure enzyme avoids this problem. In certain instances the use of enzymes becomes feasible only when they are pure like in production of low esters pectins using pure pectinesterase.

Single component retting enzyme, endopolygalacturonase such as one from *Rhizopus oryzae*, has been used effectively by Zhang and co-workers (2005) for retting flex fibres.

(c) *Evolutionary Links*: On the basis of cloning of polygalacturonase genes and other proteomic tools, Schiott and co-workers (2010) suggested that the pectinolytic enzymes from fungal symbionts of *Acromyrmex echinatior* leaf-cutting ants represent secondarily evolved adaptations that are convergent to those normally found in phytopathogens.

Conclusion

There are a number of industrial processes to which pectinases can be applied to improve the quality and yield of final products. Thus, it is important to investigate the production conditions and physicochemical characteristics of new pectinolytic enzymes for specific industrial process. Screening a large number of microorganisms for high active enzymes combined with protein engineering, direct evolution and metagenome approaches can lead to more efficient and stable enzymes. Moreover, research on the development of novel bioreactors to carry out efficient hydrolysis of pectic substrates by pectinases has not received much attention. In order to develop novel bioreactors, an understanding of the stability characteristics of pectinases is required. This poses a challenging task for protein and biochemical engineers. In the near future, biochemical techniques and innovative experiments in cellular and molecular biology could offer real breakthrough in pectinase research.

References

Abbes F, Bouaziz MA, Blecker C, Masmoudi M, Attia H, Besbes S (2011) Date syrup: effect of hydrolytic enzymes (pectinase/cellulase) on physicochemical characteristics, sensory and functional properties. LWT- Food Sci Technol 44:1827–1834

Abdel-Halim ES, Konczewicz W, Zimniewska M, Al-Deyab SS, El-Newehy MH (2010) Enhancing hydrophilicity of bioscoured flax fabric by emulsification post-treatment. Carbohydr Polym 82:195–201

Abdullah Liew AG, Sulaiman NM, Aroua MK, Megat MJ, Megat Mohd Noor MJ (2007) Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. J Food Eng 81:65–71

Aggarwal PB, Nierstrasz VA, Bouwhuis GH, Warmoeskerken MMCG (2008) Cutinase and pectinase in cotton bioscouring: an innovative and fast bioscouring process. Biocatal Biotransform 26:412–421

Ahlawat S, Battan B, Dhiman SS, Sharma J, Mandhan RP (2007) Production of thermostable pectinase and Xylanase for their potential application in bleaching of kraft pulp. J Ind Microbiol Biotechnol 34:763–770

Akin DE, Foulk JA, Dodd RB, McAlister DD (2001) Enzyme-retting of flax and characterization of processed fibers. J Biotechnol 89:193–203

Akin DE, Condon B, Sohn M, Foulk JA, Dodd RB, Rigsby L (2007) Optimization for enzyme-retting of flax with pectate lyase. Ind Crop Prod 25:136–146

Alana A, Alkorta I, Domíngez JB, Llama MJ, Serra JL (1990) Pectin lyase activity in a *Penicillium italicum* strain. Appl Environ Microbiol 56:3755–3759

Alix S, Lebrun L, Marais S, Philippe E, Bourmaud A, Baley C, Morvan C (2012) Pectinase treatment on technical fibres of flax: effects on water sorption and mechanical properties. Carbohydr Polym 87:177–185

Alkorta I, Garbisu C, Liama MJ, Serra JL (1998) Industrial applications of pectic enzymes: a review. Process Biochem 33:21–28

Almeida C, Brányik T, Moradas-Ferreira P, Teixeira J (2005) Use of two different carriers in a packed bed reactor for endopolygalacturonase production by a yeast strain. Process Biochem 40:1937–1942

Angayarkanni J, Palaniswamy M, Murugesan S, Swaminathan K (2002) Improvement of tea leaves fermentation with *Aspergillus* spp. Pectinase J Biosci Bioeng 94:299–303

Antier P, Minjares A, Roussos S, Raimbault M, Gustavo V-G (1993) Pectinase hyper-producing mutants of Aspergillus niger C28B25 for solid-state fermentation of coffee pulp. Enzyme Microb Technol 15:254–260

Armada L, Fernnadez E, Falque E (2010) Influence of several enzymatic treatments in aromatic composition of white wines. LWT- Food Sci Technol 43:1517–1525

Basu S, Saha MN, Chattopadhyay D, Chakarbati K (2009) Large scale degumming of ramie fibre using a newly isolated Bacillus pumilus DKS1 with high pectate lyase activity. J Ind Microbiol Biotechnol 36:239–245

Bauer DW, Collmer A (1997) Molecular cloning, characterization, and mutagenesis of a *pel* Gene from *Pseudomonas syringae* pv. *lachrymans* encoding a Member of the *Erwinia chrysanthemi* PelADE family of pectate lyases. Mol Plant Microbe Interact 10:369–379

Blanco P, Sieiro C, Reboredo NM, Villa TG (1998) Cloning, molecular characterization, and expression of an endo- polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. FEMS Microbiol Lett 164:249–255

Brinton CS, Dore WH, Wichmann HJ, Willaman JJ, Wilson CP (1927) Definitions written by the committee on nomenclature of pectin of the agriculture food division. J Am Chem Soc 49:38–40

Cao J, Zheng L, Chen S (1992) Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. Enzyme Microb Technol 14:1013–1016

Ceci L, Lozano J (1998) Determination of enzymatic activities of commercial pectinases for the clarification of apple juice. Food Chem 61:237–241

Cheirslip B, Unsakul K (2008) Processing of banana based wine product using pectinase and α-amylase. J Food Process Eng 31:78–90

Chen WC, Hsieh HJ, Tseng TC (1998) Purification and characterization of a pectin lyase from Pythium splendens infected cucumber fruits. Bot Bull Acad Sin 39:181–186

Chimwamurombe PM, Brenda D, Wingfield AMB, Wingfield MJ (2001) Cloning and sequence analysis of the endopolygalacturonase gene from the pitch canker fungus, *Fusarium circinatum*. Curr Microbiol 42:350–352

Cosgrove DJ (1997) Assembly and enlargement of the primary cell wall in plants. Annu Rev Cell Biol 13:171–201

Demarche P, Junghanns C, Nair RR, Agathos SN (2011) Harnessing the power of enzymes for environmental stewardship. Biotechnol Adv 30(5):933–953. http://dx.or/10.1016/j.biotechadv.2011.05.013

Demir N, Acar J, Sarioglu K, Mutlu M (2001) The use of commercial pectinase in fruit juice industry. Part 3: immobilized pectinase for mash treatment. J Food Eng 47:275–280

Deshpande KS, Gurucharanam K (1985) Degumming of ramie fibres: role of cell wall degrading enzymes of *Aspergillus versicolor*. Ind J Bot 8:79–81

Devi NA, Rao AGA (1996) Fractionation, purification and preliminary characterization of polygalacturonases produced by *Aspergillus carbonarius*. Enzyme Microb Technol 18:59–65

Diaz AB, Bolivar J, de ory I, Caro I, Blandino A (2011) Applicability of enzymatic extracts obtained by solid-state fermentation on grape pomace and orange peels mixtures in must clarification. LWT- Food Sci Technol 44:840–846

Dinnella C, Lanzarini G, Stagni A (1994) Immobilization of an endopectin lyase on g-alumina: study of factors influencing the biocatalytic matrix stability. J Chem Technol Biotechnol 59:237–241

Dixit VS, Kumar AR, Pant A, Khan MI (2004) Low molecular mass pectate lyase from *Fusarium moniliforme*: similar modes of chemical and thermal denaturation. Biochem Biophys Res Commun 315:477–484

Dzogbefia VP, Djokoto DK (2006) Combined effects of enzyme dosage and retention time on papaya juice extraction with the aid of pectic enzymes-a preliminary report. J Food Biochem 30:117–122

Esquivel JC, Voget CE (2004) Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. J Biotechnol 110:21–28

Evans JD, Akin DE, Foulk JA (2002) Flax-retting by polygalacturonase-containing enzyme mixtures and effects on fiber properties. J Biotechnol 97:223–231

Gadre RV, Driessche GV, Beeumen JV, Bhat MK (2003) Purification, characterization and mode of action of an endo-polygalacturonase from the psychrophilic fungus *Mucor flavus*. Enzyme Microb Technol 32:321–330

Ganivors A, Nedjaoum N, Gognies S, Muzart M, Nedjma M, Belarbi A (2000) Purification and characterization of acidic endo polygalacturonase encoded by the PGL-1 gene from *Saccharomyces cerevisiae*. FEMS Microbiol Lett 183:131–135

Gupta S, Kapoor M, Sharma KK, Nair LM, Kuhad RC (2008) Production and recovery of an alkaline exo-polygalacturonase from Bacillus subtilis RCK under solid-state fermentation using statistical approach. Bioresour Technol 99:937–945

Hasunuma T, Fukusaki EI, Kobayashi A (2003) Methanol production is enhanced by expression of an *Aspergillus niger* pectin methylesterase in tobacco cells. J Biotechnol 106:45–52

Hebeish A, Hashem M, Shaker N, Ramadan M, El-Sadek B, Hady MA (2009) New development for combined bioscouring and bleaching of cotton based fabrics. Carbohydr Polym 78:961–972

Hoondal GS, Tiwari RP, Tiwari R, Dahiya N, Beg QK (2002) Microbial alkaline pectinase and their applications: a review. Appl Microbiol Biotechnol 59: 409–418

Ishida Y, Kakibuchi K, Hirao Y, Izumori K (1997) Cloning and characterization of a polygalacturonase-encoding gene from *Penicillium janthinellum*. J Ferment Bioeng 84:257–260

Jacob N, Poorna AC, Prema P (2008) Purification and partial characterization of polygalacturonase from *Streptomyces lydicus*. Bioresour Technol 99:6697–6701

Jain S, Duron H, Tiraby G (1990) Production of extracellular pectinase enzymes by a mutant (PO16) of Penicillium occitanis. Appl Microbiol Biotechnol 34:308–312

Jayani RS, Saxena S, Gupta R (2005) Microbial pectinolytic enzymes a review. Process Biochem 40:2931–2949

Joshi V, Parmar M, Rana N (2011) Purification, characterization of pectinase produced from apple pomace and its evaluation in the fruit juice extraction and clarification. Indian J Nat Prod Resour 2(2):189–197

Kapoor M, Kuhad RC (2002) Improved polygalacturonase production from Bacillus sp. MG-cp-2 under submerged (SmF) and solid state (SSF) fermentation. Lett Appl Microbiol 34:317–322

Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001) Application of an alkaline and thermostable polygalacturonase from Bacillus sp, MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sun hemp (Crotalaria juncea) bast fibres. Process Biochem 36:803–807

Kareem SO, Adebowale AA (2007) Clarification of orange juice by crude fungal pectinase from citrus peel. Niger Food J 25(1)

Kashyap DR, Chandra S, Kaul A, Tewari R (2000) Production, purification and characterization of pectinase from a *Bacillus* sp. DT7. World J Microbiol Biotechnol 16:277–282

Kashyap DR, Vohra PK, Chopra S, Tiwari R (2001) Applications of pectinases in the commercial sector: a review. Bioresour Technol 77:215–227

Kaur G, Satyanarayana T (2004) Production of extracellular pectinolytic, cellulolytic and xylanolytic enzymes by thermophilic mould Sporotrichum thermophile *Apinis* in solid-state fermentation. Indian J Biotechnol 3:552–557

Kaur A, Mahajan R, Singh A, Garg G, Sharma J (2010) Application of cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in biobleaching of kraft pulp. Bioresour Technol 101:9150–9155

Kavitha R, Kumar-Umesh S (2000) Genetic improvement of *Aspergillus carbonarius* for pectinase overproduction during solid-state growth. Biotechnol Bioeng 67(1):121–125

Kelebek H, Canbas A, Carbaroglu A, Selli S (2007) Improvement of anthocyanin content in the cv. Okuzgozu wines by using pectinolytic enzymes. Food Chem 105(1):334–339

Kitamoto N, Kimura T, Kito Y, Ohmiya K, Tsukagoshi N (1993) Structural features of polygalacturonase gene cloned from *Aspergillus oryzae* KBN616. FEMS Microbiol Lett 111:37–41

Klug-Santner BG, Schnitzhofer W, Vrsanská M (2006) Purification and characterization of a new bioscouring pectate lyase from *Bacillus pumilus* BK2. J Biotechnol 121:390–401

Kobayashi T, Hatada Y, Higaki N (1999) Enzymatic properties and deduced amino acid sequence of a high-alkaline pectate lyase from an alkaliphilic *Bacillus* isolate. Biochim Biophys Acta 1427:145–154

Kuhad RC, Kapoor M, Rustagi R (2004) Enhanced production of alkaline pectinase from *Streptomyces* sp. RCK-SC by whole cell immobilization and solid-state cultivation. World J Microbiol Biotechnol 20:257–263

Lahiani HT, Ayadi M, Hadj-Taieb N, Ali MB, Gargouri A (2008) Genomic organisation of a polygalacturonase gene from a hyperpectinolytic mutant strain of *Penicillium occitanis*. FEMS Microbiol Lett 281:23–29

Landbo A-K, Meyer AS (2004) Effects of different enzymatic maceration treatments on enhancement of anthocyanins and other phenolics in black currant juice. Innov Food Sci Emerg Technol 5:503–513

Lang C, Dornenburg H (2000) Perspectives in the biological function and the technological application of polygalacturonases. Appl Microbiol Biotechnol 53:366–375

Latif S, Diosady LL, Anwar F (2008) Enzyme-assisted aqueous extraction of oil and protein from canola (Brassica napus L.) seeds. Eur J Lipid Sci Technol 110:887–892

Lee WC, Yusof S, Hamid NSA, Bahrain BS (2006) Optimizing conditions for enzymatic clarification of banana juice using response surface methodology (RSM). J Food Eng 73:55–63

Lieu LN, Le VVM (2010) Application of ultrasound in grape mash treatment in juice processing. Ultrason Sonochem 17:273–279

Limberg G, Korner R, Buchholt HC, Christensen TM, Roepstorff P, Mikkelsen JD (2000) Analysis of different de-esterification mechanisms for pectin by enzymatic fingerprinting using endopectin lyase and endopolygalacturonase II from *A. niger*. Carbohydr Res 327:293–307

Louw C, LaGrange D, Pretorius IS, VonRosenburg P (2006) The effect of polysaccharide-degrading wine yeast transformants on the efficiency of wine processing and wine flavour. J Biotechnol 125:447–461

Maciel MHC, Herculano PN, Porto TS, Teixeira MFS, Moreira KA, Souza-Molta CM (2011) Production and characterization of pectinases from forage palm by *Aspergillus niger* URM4645. Afr J Biotechnol 10(13):2469–2475

Maleki MH, Tajick Ghanbary MA, Ranjbar G, Asgharzadeh A, Lotfi A (2011) Screening of some zygomycetes strain for pectinase activity. J Microbiol Biotechnol Res 1:1–7

Malvessi E, Silveira MM (2004) Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus oryzae*. Braz Arch Biol Technol 47:693–702

Markowski J, Mieszczakowska M, Pocharski W (2009) Effect of apple cultivar and enzyme treatment on phenolic compounds content during clear apple juice production. Int J Food Sci Technol 44:1002–1010

Masoud W, Jespersen L (2006) Pectin degrading enzymes in yeasts involved in fermentation of coffea arabica in East Africa. Int J Food Microbiol 110:291–296

Miyazaki Y (1991) Purification and characterization of an endo-pectate lyase from *Bacillus macerans*. Agric Biol Chem 55:23–30

Moreau RA, Johnston DB, Powell MJ, Hicks KB (2004) A comparison of commercial enzymes for the aqueous enzymatic extraction of corn oil from corn germ. JAOCS 81(11):1071–1075

Morozova VV, Semenova MV, Salanovich TN (2006) Application of neutral-alkaline pectate lyase to cotton fabric boil off. Appl Biochem Microbiol 42:603–608

Murad HA, Azzaz HH (2011) Microbial pectinases and ruminant nutrition. Res J Microbiol 6:246–269

Nadaroglu H, Taskin E, Adiguzel A, Medine G, Demir N (2010) Production of a novel pectin lyase from *Bacillus pumilus* (P9), purification and characterization and fruit juice application. Rom Biotechnol Lett 15:5167–5176

Nakamura M, Hisashi IWA, Kei ARAP (2002) Cloning and characterization of a polygalacturonase gene *Ap2pgl* from *Geotrichum candidum* citrus race Ap2 pathogenic to apple fruit. J Gen Plant Pathol 68:333–337

Nakkeerran E, Kumar-Umesh S, Subramaniam R (2011) *Aspergillus carbonarius* polygalacturonases purified by integrated membrane process and affinity precipitation for apple juice production. Bioresour Technol 102:3293–3297

Ogawa A, Sawada K, Saito K, Hakamada Y, Sumitomo N, Hatada Y, Kobayashi T, Ito S (2000) A new high-alkaline and high-molecular-weight pectate lyase from a *Bacillus* isolate: enzymatic properties and cloning of the gene for the enzyme. Biosci Biotechnol Biochem 64:1133–1141

Oliveira KF, Malavolta L, Souza CS, Vionte EJ, Laluca C (2006) Pectinolytic activity secreted by yeasts isolated from fermented citrus molasses. J Appl Microbiol 100:633–640

Pagan A, Conde J, Ibarz A, Pagan J (2006) Orange peel degradation and enzyme recovery in the enzymatic peeling process. Int J Food Sci Technol 41:113–120

Pagès S, Heijne WHM, Kester HCM, Visser J, Benen JAE (2000) Subsite mapping of *Aspergillus niger* endopolygalacturonase II by site-directed mutagenesis. J Biol Chem 275:29348–29353

Parenicová L, Benen JAE, Kester HCM, Visser J (1998) *pga*E encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. Eur J Biochem 251:72–80

Pedrolli DB, Gomes E, Monti R, Carmona EC (2008) Studies on productivity and characterisation of polygalacturonase from Aspergillus giganteus submerged culture using citrus pectin and orange waste. Appl Biochem Biotechnol 144(2):191–200

Pedrolli DB, Monteiro AC, Gomes E, Carmona CE (2009) Pectin and pectinases: production, characterization and industrial application of microbial pectinolytic enzymes. Open Biotechnol J 3:9–18

Pinelo M, Zeuner B, Meyer AS (2010) Juice clarification by protease and pectinase treatments indicate new roles of pectin and protein in cherry juice turbidity. Food Bioprod Process 88:259–265

Prathyusha K, Suneetha V (2011) Bacterial pectinases and their potent biotechnological application in fruit processing/juice production industry: a review. J Phytol 3:16–19

Pretel MT, Botella MA, Amoros A, Zapata PJ, Serreno M (2007a) Optimization of vacuum infusion and incubation time for enzymatic peeling of Thomson and Mollar oranges. LWT- Food Sci Technol 40:12–22

Pretel MT, Botella MA, Amoro A, Zapata PJ, Serrano M (2007b) Optimization of vacuum infusion and incubation time for enzymatic peeling of Thompson and Mollar oranges. LWT 40:12–20

Rai P, Majumdar GC, DasGupta S, De S (2007) Effect of various pre-treatment methods on permeate flux and quality during ultrafiltration of mosambi juice. J Food Eng 78:561–568

Rasheedha Banu A, Kalpana Devi M, Gnanaprabhal GR, Pradeep BV, Palaniswamy M (2010) Production and characterization of pectinase enzyme from *Penicillium chrysogenum*. Indian J Sci Technol 3:377–381

Rashmi R, Siddalinga K, Sneha G, Shabana S, Syama A, Radhik V (2008) Partial purification and biochemical characterization of extracellular pectinase from *Aspergillus niger* isolated from groundnut seeds. J Appl Biosci 9:378–384

Reid I, Ricard M (2000) Pectinase in papermaking: solving retention problems in mechanical pulps bleached with hydrogen peroxide. Enzyme Microb Technol 26:115–123

Rexova-Benkova L, Markovie O (1976) Pectic enzymes. Adv Carbohydr Chem 33:323–385

Ribiero DS, Henrique SMB, Oliviera LS, Macedo GA, Fleuri LF (2010) Enzymes in juice processing: a review. Int J Food Sci Technol 45:635–641

Said S, Fonseca MJV, Siessere V (1991) Pectinase production by Penicillium frequentans. World J Microbiol Biotechnol 7:607–608

Saito K, Takakuwa N, Oda Y (2004) Purification of the extracellular pectinolytic enzyme from the fungus *Rhizopus oryzae* NBRC 4707. Microbiol Res 159:83–86

Sakai T, Sakamoto T, Hallaert E, Vandamme EJ (1993) Pectin, pectinase and protopectinase: production, properties and applications. Adv Appl Microbiol 39:213–294

Saleem Z, Rennebaun H, Pudel F, Grimm E (2008) Treating bast fibres with pectinase improves mechanical characteristics of reinforced thermoplastic composites. Compos Sci Technol 68:471–476

Sandri IG, Fontana RC, Barfknecht DM, Silveira MM (2011) Clarification of fruit juices by fungal pectinases. LWT- Food Sci Technol 44:2217–2222

Sawada K, Ueda M (2001) Enzyme processing of textiles in reverse micellar solution. J Biotechnol 89:263–269

Sawada K, Suzumatsu A, Kobayashi T, Ito S (2001) Molecular cloning and sequencing of the gene encoding an exopolygalacturonase of a *Bacillus* isolate and properties of its recombinant enzyme. Biochimica et Biophysica Acta 1568:162–170

Schiott M, Rogowska-Wrzesinska A, Roepstroff P, Boomsma JJ (2010) Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. BMC Biol 8:156

Semenova MV, Grishutin SG, Gusakov AV, Okunev ON, Sinitsyu AP (2003) Isolation and properties of pectinases from the fungus *Aspergillus japonicus*. Biochemistry 68:559–569

Sharma DC, Satyanarayana T (2006) A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* dcrl in submerged fermentation by using statistical methods. Bioresour Technol 97:727–733. J Food Eng 73:313–319

Sharma HSS, Van Sumere CF (1992) Enzyme treatment of flax. Genet Eng Biotechnol 12:19–23

Sharma S, Mandhan RP, Sharma J (2011) *Pseudozyma* sp. SPJ: an economic and eco-friendly approach for degumming of flax fibres. World J Microbiol Biotechnol 27:2697–2701

Sieiro C, Poza M, Vilanora M, Villa TG (2003) Heterologous expression of the *Saccharomyces cerevisiae* PGU1 gene in Schizosaccharomyces pombe yields an enzyme with more desirable properties for the food industry. Appl Environ Microbiol 69:1861–1865

Sin HN, Yusof S, Sheikh Abdul Hamid N, Rahman RA (2006) Optimization of enzymatic clarification of sapodilla juice using response surface methodology. J Food Eng 73:313–319

Singh S, Gupta R (2003) Apple juice clarification using fungal pectinolytic enzyme and gelatin. Indian J Biotechnol 3:573–576

Singh SA, Plattner H, Diekmann H (1999) Exo polygalacturonate lyase from a thermophilic *Bacillus* sp. Enzyme Microb Technol 25:420–425

Soriano M, Diaz P, Pastor FIJ (2006) Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin. Curr Microbiol 50:114–118

Sriroth K, Chollakup R, Chotineeranat S, Piyachomkwan K, Oates CG (2000) Processing of Cassava waste for improved biomass utilization. Bioresour Technol 71:63–69

Suykerbuyk ME, Schaap PJ, Stam H, Musters W, Visser J (1995) Cloning, sequence and expression of the gene coding for rhamnogalacturonase of *Aspergillus aculeatus*; a novel pectinolytic enzyme. Appl Microbiol Biotechnol 43:861–870

Teixeira JA, Goncalves DB, de Queiroz MV, de Araujo EF (2011) Improved pectinase production in *Penicillium griseoroseum* recombinant strains. J Appl Microbiol. d oi:10.1111/j.1365-2672.2011.05099

Toker I, Bayindirli A (2003) Enzymatic peeling of apricots, nectarines and peaches. LWT 36:215–221

Torres EF, Sepulveda TV, Gonzalez GV (2006) Production of hydrolytic depolymerising pectinases. Food Technol Biotechnol 44:221–227

Tran PPT, Le VVM (2011) Effects of ultrasound on catalytic efficiency of pectinase preparation during the treatment of pineapple mash in juice processing. Int Food Res J 18:347–354

Truong LV, Tuye H, Helmke E, Binh LT, Schweder T (2001) Cloning of two pectate lyase genes from the marine Antarctic bacterium Pseudoalteromonas haloplanktis strain ANT/505 and characterization of the enzymes. Extremophiles 5:35–44

Unejo M, Pastore GM (2007) Pectinases: industrial applications and perspectives. Quim Nova 30:388–394

Van Sumere CF (1992) Retting of flax with special reference to enzyme retting. In: Sharma HSS, Van Sumere CF (eds) The biology and processing of flax. M. Publications, Belfast, pp 157–198

Van Sumere CF, Sharma HSS (1991) Analyses of fine flax fibre produced by enzymatic retting. Asp Appl Biol 28:15–20

Vierhuis E, Servili M, Baldioli M, Schols HA, Voragen AGJ, Montedoro GF (2001) Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. J Agric Food Chem 49:1218–1223

Viikari L, Tenkanen M, Suurnakki A (2001) Biotechnology in the pulp and industry. In: Rehm HJ (ed) Biotechnology, vol 2. Wiley VCH, Chichester, pp 523–546

Vijyanand P, Kulkarni SG, Prathibha GV (2010) Effect of pectinase treatment and concentration of litchi juice on quality characteristics of litchi juice. J Food Sci Technol 47:235–239

Vlugt-Bergmans C, Vander JB, Meeuwsen PJA, Voragen AGJ (2000) Endo-xylogalacturonan hydrolase, a novel pectinolytic enzyme. Appl Environ Microbiol 33:36–41

Walker SG, Ryan ME (2003) Cloning and expression of a pectate lyase from the oral spirochete *Treponema pectinovorum* ATCC 33768. FEMS Microbiol Lett 226:385–390

Wang Q, Fan X-R, Hua Z-Z, Chen J (2007) Optimizing bioscouring condition of cotton knitted fabrics with an alkaline pectinase from *Bacillus subtilis* WSHB04-02 by using response surface methodology. Biochem Eng J 34:107–113

Whitaker JR (1990) Microbial pectinolytic enzymes. Microb Enzyme Biotechnol 2:133–176

Whitehead MP, Shieh MT, Cleveland TE, Cary JW, Dean RA (1995) Isolation and characterization of polygalacturonase genes (*pecA* and *pecB*) from *Aspergillus flavus*. Appl Environ Microbiol 61:3316–3322

Wubben JP, Mulder W, Ten Have A, Van Kan JA, Visser J (1999) Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. Appl Environ Microbiol 65:1596–1602

Yadav S, Yadav PK, Yadav D, Deo K, Yadav S (2008) Purification and characterization of an alkaline pectin lyase from *Aspergillus flavus*. Process Biochem 43:547–552

Yuan P, Meng K, Huang H, Shi P, Luo H, Yang P, Yao B (2011) A novel acidic and low-temperature-active

endo-polygalacturonase from Penicillium sp. CGMCC 1669 with potential for application in apple juice clarification. Food Chem 129:1369–1375

Zhai C, Cao J, Wang Y (2003) Cloning and expression of a pectate lyase gene from *Bacillus alcalophilus* NTT33. Enzyme Microb Technol 33(2):173–178

Zhang J, Henriksson H, Szabo IJ, Henriksson G, Johansson G (2005) The active component in the flax-retting system of the zygomycete Rhizopus oryzae sp. is a family 28 polygalacturonase. J Ind Microbiol Biotechnol 32:431–438

Zhao Q, Yuan S, Zhang Y, Zhu H, Dai C, Yang F, Fengmin H (2007) Expression, purification and characterization of pectate lyase A from *Aspergillus nidulans* in *Escherichia coli*. World J Microbiol Biotechnol 23(8):1057–1064

Zhuge B, Du GC, Shen W, Zhuge J, Chen J (2008) Expression of a *Bacillus subtilis* pectate lyase gene in *Pichia pastoris*. Biochem Eng J 40:92–98

Biofuels: The Environment-Friendly Energy Carriers

8

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Abstract

Escalating globalisation, high demand for energy, increasing greenhouse gas emissions and depleting fossil fuel reserves have necessitated the search for alternative and sustainable energy carriers such as biofuels. Worldwide, the laboratories are engaged in extensive research for the development of different biofuels such as bioethanol, biodiesel, biohydrogen, biogas and advanced bioalcohols. This chapter provides an overview of bioprocessing of various types of biofuels.

Keywords

Alternative energy • Biofuels • GHG emissions • Bioalcohols • Renewable energy

Introduction

The constant increase in the utilisation of energy in transport and industrial sectors has led to an ever increasing pressure on the availability and cost of existing fossil fuels system. Also, increase in general awareness of environmental issues such as global warming and consequences of greenhouse gas (GHG) emissions, and continuous depletion of fossil fuel reserves have further initiated exploration of alternative resources of energy. Several agencies are looking upon biofuels, the environment-friendly alternative energy carriers to the petrochemical-based transportation fuels.

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Table 8.1 Benefits of biofuels

Economic impacts	Sustainability
	Fuel diversity
	Increased number of rural manufacturing jobs
	Increased income
	Increased investments in plant and equipment
	Agricultural development
	International competitiveness
	Reducing the dependency on imported petroleum
Environmental impacts	Greenhouse gas reductions
	Reducing of air pollution
	Biodegradability
	Higher combustion efficiency
	Improved land and water use
	Carbon sequestration
Energy security	Domestic targets
	Supply reliability
	Reducing use of fossil fuels
	Ready availability
	Domestic distribution
	Renewability

Biofuel, any fuel made from organic matter resulting from agriculture or forestry, is a multiple objective sustainable resource, promising to substitute fossil fuels with energy from agricultural sources while providing a range of other benefits (Lovett et al. 2011). Currently, several types of biofuels, viz. bioethanol, biodiesel, biohydrogen, biomethane, biomethanol, biopropanol and biobutanol, are under consideration as potential alternative to fossil fuels.

Earlier, the main feedstocks for biofuels were food crops (first-generation biomass); however, the complete dependence of first-generation biofuels on food crops has caused food vs. fuel competition. However, shifting the dependence on secondary agriculture biomass has shown potential to be used as a feedstock for the production of biofuels, which otherwise would be treated as waste. Thus, biofuels can also help to reduce waste as well as providing a source of fuel. As second-generation biofuels technologies advance, it will become a preferable source of energy to both first-generation biofuels and fossil fuels, because of its wide range of benefits (Table 8.1). Second-generation biofuels does not depend on a particular feedstock and does not require highly fertile land for agriculture. As biofuels begin to enjoy growing acceptance around the world and in international markets, they could lower down the problems of energy supply as well as GHG emissions.

Bioethanol

Bioethanol is the most widely accepted biofuel to be used as an alternative to the gasoline. Currently, the major producers of ethanol are Brazil and the United States, where it is produced from sugarcane juice and corn grain, respectively. In India, ethanol is mainly produced from sugarcane molasses.

The bioconversion of secondary agricultural biomass to ethanol consists of two main processes: hydrolysis of lignocellulosic carbohydrate to fermentable reducing sugars and fermentation of the sugars to ethanol (Fig. 8.1). The hydrolysis of biomass is usually catalysed by cellulase, and the fermentation is carried out by yeasts or bacteria. The main factors that affects cellulose hydrolysis are porosity, crystallinity, and lignin and hemicellulose content

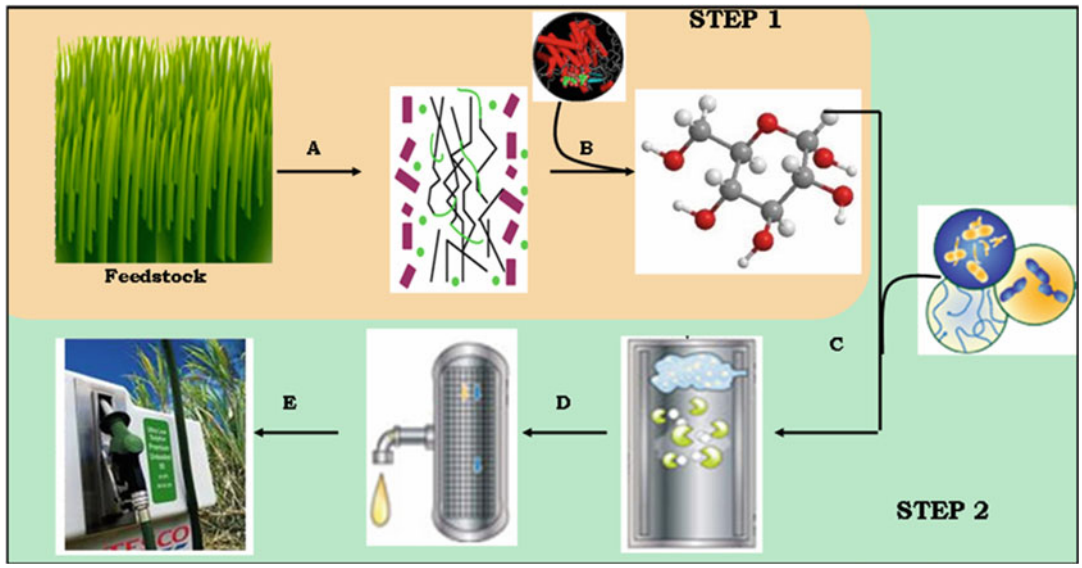


Fig. 8.1 Schematic overview of bioethanol production process (**a** pretreatment, **b** enzymatic saccharification, **c** fermentation, **d** distillation, **e** blending)

(Margeot et al. 2009; Alvira et al. 2010; Kuhad et al. 2011). The presence of lignin and hemicellulose in lignocellulosic materials reduces the hydrolysis efficiency (Himmel et al. 2007). Pretreatment of lignocellulosic biomass before hydrolysis can significantly improve the hydrolysis efficiency by removal of lignin and hemicellulose, reduction of cellulose crystallinity and increase in porosity (Mosier et al. 2005; Kumar et al. 2009; Kuhad et al. 2011). Pretreatment can be carried out in different ways such as mechanical comminuting, steam explosion, ammonia fibre explosion, and acid or alkaline and biological pretreatments (Gupta et al. 2009, 2011; Kumar et al. 2009; Kuhad et al. 2010a).

Enzymatic hydrolysis of cellulose by cellulases is highly specific, and the major product of the hydrolysis is glucose. The utility cost of enzymes is lower than acid or alkaline hydrolysis because it is usually conducted at mild conditions and has no corrosion of equipment (Kuhad et al. 2010b). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. Of all these organisms, *Trichoderma* has been the most widely studied for cellulase production. The cellulase system contains three major enzyme components: endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glycosidase (EC 3.2.1.21). All these cellulase components act in a synergistic manner (Boraston et al. 2004; Kuhad et al. 2010b). The exoglucanase (CBH) acts on the ends of the cellulose chain and releases β-glucosidase as the end-product; the EG randomly attacks the internal *O*-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glycosidases act specifically on the cellobiose disaccharides and produce glucose (Kuhad et al. 2010b). Hemicellulases are another group of polysaccharide-degrading enzymes that are specific to the hemicellulose substrate.

Biological ethanol fermentation is a process in which sugars are fermented by microorganisms to produce ethanol and CO_2. Compared to starch and molasses, the fermentation of lignocellulosic hydrolysates is a more complicated process. Two major steps of ethanol fermentation from lignocellulosic biomass involve chemical and enzymatic hydrolysis, where the enzymatic hydrolysis of biomass releases the hydrolysate that contains mostly hexose sugars, but the acid hydrolysis yields not only pentose and hexose sugars but also a few fermentation inhibitors, such as furans and phenolics. Thus, detoxification methods are required to

Table 8.2 Ethanol-producing microorganisms from hexose and pentose sugars

Pentose-fermenting microbes	References	Hexose-fermenting microbes	References
Aeromonas hydrophila	Singh and Mishra (1993)	*Candida shehatae*	Abbi et al. (1996a)
Bacillus macerans	Dien et al. (2003)	*Fusarium sporium*	Mamma et al. (1995)
Bacillus polymyxa	Singh and Mishra (1993)	*Kloeckera apiculata*	Aguilera et al. (2006)
Bacteroides polygramatis	Patel (1984)	*Kluyveromyces marxianus*	Ballesteros et al. (2004)
Clostridium acetobutylicum	El Kanouni et al. (1998)	*Mucor indicus*	Abdenifar et al. (2009)
Clostridium thermocellum	Herrero and Gomez (1980)	*M. hiemalis*	Millati et al. (2005)
Escherichia coli	Yomano et al. (1998)	*M. corticolous*	Millati et al. (2005)
Klebsiella oxytoca	Ingram et al. (1999)	*Neurospora crassa*	Mamma et al. (1995)
Lactobacillus pentosus	Chaillou et al. (1999)	*Pachysolen tannophillus*	Abbi et al. (1996a)
Lactobacillus casei	Roukas and Kotzekidou (1998)	*Pichia stipitis*	Gupta et al. (2009)
Lactobacillus pentoaceticus	Chaillou et al. (1999)	*Pichia membranifaciens*	Aguilera et al. (2006)
Lactobacillus plantarum	Sreenath et al. (1999)	*Rhizopus oryzae*	Abdenifar et al. (2009)
Lactobacillus xylosus	Sreenath et al. (1999)	*Rhizopus miehei*	Millati et al. (2005)
Candida boidinii	Vandeska et al. (1996)	*Rhizomucor pusillus*	Millati et al. (2005)
Candida shehatae	Abbi et al. (1996a)	*Saccharomyces cerevisiae*	Kuhad et al. (2010b)
Fusarium oxysporum	Jeffries and Jin (2004)	*S. bayarus*	Belloch et al. (2008)
Mucor corticolous	Millati et al. (2005)	*S. paradoxus*	Belloch et al. (2008)
Mucor hiemalis	Millati et al. (2005)	*S. kudriavzevii*	Belloch et al. (2008)
Mucor indicus	Millati et al. (2005)	*S. cariocanus*	Belloch et al. (2008)
Neurospora crassa	Deshpande et al. (1986)	*S. mikatae*	Belloch et al. (2008)
Pachysolen tannophilus	Schneider et al. (1981)	*S. pastorianus*	Belloch et al. (2008)
Paecilomyces sp. *NF1*	Mountfort and Rhodes (1991)	*Schizosaccharomyces pombe*	Hu et al. (2005a, b)
Pichia stipitis	Gupta et al. (2009)	*Torulaspora delbrueckii*	Aguilera et al. (2006)

improve the fermentability of acid hydrolysate, for example, overliming, ion-exchange adsorption, activated carbon adsorption, solvent extraction, steam stripping and enzymatic (laccase) treatments (Chandel et al. 2007; Mosier et al. 2005; Palmqvist and Hahn-Hägerdal 2000a, b).

A variety of microorganisms ranging from bacteria, fungi and yeasts are known to ferment hexose sugars (Table 8.2). The most common and efficient microbe used for hexose fermentation is *Saccharomyces cerevisiae* (Hahn-Hägerdal et al. 2007). Various studies have been carried out using *S. cerevisiae* for the fermentation of lignocellulosic hydrolysates (Lee et al. 1999; Wang et al. 2004; Chen et al. 2007; Rocha et al. 2009; Gupta et al. 2009; Kuhad et al. 2010a). As an additional approach, simultaneous saccharification and fermentation (SSF) process has also been used for improved ethanol production. In the SSF process, the stages are virtually the same as in separate hydrolysis and fermentation systems, except that both are performed in the same reactor. It has been shown that SSF reduces the processing time, which, in turn, leads to an increase in the productivity of ethanol (Alfani et al. 2000; Soderstrom et al. 2005; Ohgren et al. 2007). Further, in order to economise the ethanol production, both pentose and hexose sugars must be converted to ethanol; however, even the most promising fermenting microbes do not efficiently ferment pentoses. Among the most common yeast species identified so far for the pentose fermentation are *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* (Abbi et al. 1996a, b; Hahn-Hägerdal et al. 2007; Mosier et al. 2005; Palmqvist and Hahn-Hägerdal 2000a; Talebnia et al. 2008). Some other microorganisms that can ferment pentose sugars are listed in Table 8.2.

Although *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* can ferment pentose sugars, their commercial exploitation for ethanol production is limited because of their low ethanol tolerance, slow rates of fermentation, difficulty in controlling the rate of oxygen supply and sensitivity to inhibitors generated during pretreatment and hydrolysis of lignocellulosic substrates (Hahn-Hägerdal et al. 2007; Kumar et al. 2009). In mixed sugar fermentation, the pentose uptake is inhibited by hexoses, and thus, the pentose fermentation is only possible at very low glucose concentrations. In the last decade, genetic engineering of microorganisms used in ethanol production has shown significant progress (Jeffries and Jin 2004). Besides *S. cerevisiae*, bacteria such as *Zymomonas mobilis* and *Escherichia coli* have been targeted through metabolic engineering for ethanol production from lignocellulosic biomass (Jeffries 2006; Karhumaa et al. 2005, 2007; Liu and Hu 2010; Matsushika et al. 2008; Runquist et al. 2009; Yang et al. 2009). Few potent recombinant microbes with bioethanol potential are listed in Table 8.3.

The first techno-economic study was carried out in 1987 by the US National Renewable Energy Laboratory (NREL). The second economical study was carried out in 2002 by Aden and coworkers. As an estimation by the Energy Information Administration (EIA 2009), the wholesale price of gasoline in 2012 will be $2.62/gal of gasoline (US$ of 2007). Assuming a conversion factor of 0.67 gal of gasoline per gallon of ethanol, the projected cost of ethanol is set at $1.76/gal of ethanol (US dollar of 2007). However, the ethanol cost projection of the *n*th ethanol plant is at $1.49/gal of ethanol (US dollar of 2007).

Besides the USA, European research institutions, mainly in Sweden, the Netherlands and Denmark, have also evaluated the economics of bioethanol production. In the REFUEL project, 7 European institutes have analysed biofuels in terms of resource potential, costs and impacts. The data for bioethanol production from cellulosic materials based on the enzymatic hydrolysis strategy was procured from the Energy Research Centre of the Netherlands (Kuijvenhoven 2006) and the Copernicus Institute for Sustainable Development and Innovation of Utrecht University (Hamelinck 2004). The evaluation (Londo et al. 2008) resulted in a net production cost (including the sales of electricity as a by-product) of €0.62/L in 2010, €0.59/L in 2020 and €0.50/L in 2030.

Now it has been understood that the increasing production capacity to commercial scale can only be done with confidence when a process is shown to be robust at an intermediate, pilot scale. An ideal pilot plant needs to be fully integrated and should be able to evaluate the complete system (e.g., enzymes and yeasts) while having sufficient flexibility to investigate alternative process configurations and test opinions for better heat integration and recycling of process streams.

Biodiesel

Biodiesel is defined as the alkyl esters of vegetable oils or animal fats. The vegetable oils and fats can be considered as alternative source of transportation fuels with viscosities ranging from 10 to 17 times higher than the existing fossil fuel. Biodiesels have properties closer to gasoline and can be blended at high levels in certain vehicles. Currently, biodiesel-powered flexible-fuel vehicles are widely available in many countries (Carraretto et al. 2004).

Biodiesel is produced by transesterification of plant oil or fat to achieve a viscosity close to that of petroleum diesel. The conversion has two main steps, transesterification and hydrogenation. Transesterification refers to a reaction between triglyceride of one alcohol and a second alcohol to form an ester of the second alcohol (methyl ester). Transesterification of oils and fats to generate esters and glycerin is a well-established process. The purpose of the transesterification process is to lower the viscosity of the oil. While hydrogenation produces renewable diesels of superior quality and free of particulates and by-products (Gerhard 2010).

Various studies have been investigated using several oils as well as different catalysts such as

Table 8.3 List of pentose utilising recombinant yeasts and bacterial strains

Strain	Ethanol yield (g/g sugar)	Ethanol productivity (g/L/h)	References
E. coli KO11	102	0.87	Ohta et al. (1991)
E. coli KO11	89	0.85	Yomano et al. (1998)
E. coli FBR5	90	0.59	Dien et al. (2000)
E. coli FBR5	90	0.92	Dien et al. (2000)
E. coli LY01	88	0.66	Yomano et al. (1998)
K. oxytoca M5A1	95	0.96	Ohta et al. (1991)
K. oxytoca P2	84	0.35	Bothast et al. (1994)
Z. mobilis CP4	86	0.57	Zhang et al. (1995)
Z. mobilis CP4	95	0.81	Zhang et al. (1995)
Z. mobilis ATCC 39767	82–84	0.82–0.65	Chou et al. (1997)
S. cerevisiae A4	38	NA	Zaldivar et al. (2002)
S. cerevisiae 1400	6	NA	Ho et al. (1998)
S. cerevisiae RBW 202-AFX	84	NA	Kuyper et al. (2004)
S. cerevisiae RBW 202-AFX	78	NA	Madhavan et al. (2009)
S. cerevisiae RWB 217	86	NA	Kuyper et al. (2005)
S. cerevisiae H158	44	NA	Johansson et al. (2001)
S. cerevisiae H158	54	NA	Johansson et al. (2001)
S. cerevisiae H 2673	46	NA	Verho et al. (2003)
S. cerevisiae H 2723	48	NA	Verho et al. (2003)
S. cerevisiae H 2684	82	NA	Verho et al. (2003)
S. cerevisiae ZU-10	75.6	0.50	Zhao and Xia (2010)
S. cerevisiae LEK 122	24.5	0.025	Liu and Hu (2010)
S. cerevisiae LEK 122	25.4	0.064	Liu and Hu (2010)
S. cerevisiae LEK 513	32.5	0.113	Liu and Hu (2010)
S. cerevisiae TMB 3001	25	0.15	Eliasson et al. (2000)
S. cerevisiae TMB 3001	62	0.22	Jeppsson et al. (2002)
S. cerevisiae TMB 3008	76	0.27	Jeppsson et al. (2002)
S. cerevisiae TMB 3030	56	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3037	68	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3050	58	NA	Karhumaa et al. (2005)
S. cerevisiae TMB 3057	66	0.13	Karhumaa et al. (2007)
S. cerevisiae TMB 3066	86	0.073	Karhumaa et al. (2007)
S. cerevisiae TMB 3251	68	0.24	Jeppsson et al. (2002)
S. cerevisiae TMB 3253	56	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3254	56	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3255	82	0.29	Jeppsson et al. (2002)
S. cerevisiae TMB 3256	72	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3261	68	NA	Jeppsson et al. (2003)
S. cerevisiae TMB 3270	72	0.32	Jeppsson et al. (2006)
S. cerevisiae TMB 3271	62	0.28	Jeppsson et al. (2006)
S. cerevisiae TMB 3400	36	NA	Wahlbom et al. (2003)
S. cerevisiae TMB 3400	50	NA	Wahlbom et al. (2003)
S. cerevisiae TMB 3400	68	0.12	Karhumaa et al. (2007)
P. stipitis FPL UC7	76	0.41	Shi et al. (1999)
P. stipitis FPL-shi 21	96	0.43	Shi et al. (1999)
P. stipitis FPL-shi 31	62	0.15	Shi et al. (2002)

Table 8.4 Lipid content of selected microalga species

Microalgae species	Lipid content (% dw)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

NaOH, KOH, H_2SO_4 and supercritical fluids and lipase enzyme (Marchetti et al. 2007). Among various oil-bearing crops, only soybean, palm, sunflower, safflower, cottonseed, rapeseed and peanut oils are considered as potential for biodiesel (Goering et al. 1982; Pryor et al. 1982). However, any vegetable oil could be used for biodiesel production (Demirbas 2006). Moreover, other sources for biodiesel include fats and waste oils. Efforts have also been made to exploit various algal species that produce oils (Nagel and Lemke 1990). In addition to oils, fatty acids can also serve as a potential reactant for biodiesel production. Since fatty acid biosynthesis is a natural energy storage molecule in microorganisms, it can be further esterified in vivo to form fatty acid ethyl esters (FAEEs) known as microdiesels with similar properties to biodiesels (Kalscheuer et al. 2006). Such a production pathway has been demonstrated at pilot scale (Elbahloul and Steinbohel 2010; Steen et al. 2010).

Similar to first-generation feedstocks for bioethanol, though production of biodiesel from vegetable oils is a potential and inexhaustible source of energy with energy content close to diesel fuel, the extensive use of vegetable oils may cause its shortage in developing countries. Alternatively, a variety of biolipids can also be used for biodiesel production. These are (a) vegetable oil plants, (b) waste vegetable oil and (c) nonedible oils. In few countries like Malaysia and Indonesia, palm oil has been used for biodiesel production. While, in Europe, Rape seed is the main oil resource, in India and Southeast Asia, the Jatropha seeds have been used for biodiesel production.

In addition to the land oil crops, algae represent an important and novel platform used for biodiesel production. The algae have several advantages such as rapid growth rate, high photosynthetic efficiency and high biomass production. The use of waste nutrients is an important factor for sustainable production of algal biodiesel. Wastewater rich in N and P can be used for algae cultivation (Demirbas and Demirbas 2011). The use of residual algal biomass after lipid extraction, for example, as feed (because of the high vitamin content), is a key factor in biorefinery concepts in order to improve economic feasibility (Table 8.4). Therefore, the R&D efforts should be focused on the improvement of cost-effectiveness and sustainability for the production of biodiesel. There are numerous algal species which have potential to be used as suitable candidate for oil production (Table 8.5).

The most significant difference between algal oil and other oils is in their yield. The yield (per acre) of algae oil is approximately 200-folds higher than the plant/vegetable oils (Sheehan et al. 1998). It has been estimated that a diatom algae can produce 46 t of oil/ha/year. However, no commercial scale plant has been developed on algal biodiesel so far in India and should be seriously considered by the government and the private industry.

Similar to bioethanol, the cost of biodiesel production varies significantly, depending on the feedstock source and the scale of the plant. A review of several economic evaluation studies showed that the costs of biodiesel ranged from US$0.30–0.69/L. Rough estimations of the cost of biodiesel from vegetable oil and waste grease are US$0.54–0.62/L and US$0.34–0.42/L, respectively. With pre-tax diesel priced at US$0.18/L in the US and US$0.20–0.24/L in some European countries, biodiesel is thus currently not economically feasible, and more research

Table 8.5 List of algae used for the production of biodiesel

Name	References
Chlorella protothecoides	Nigam and Singh (2011), Mata et al. (2010) and Brennan and Owende (2010)
Spirulina platensis	Mata et al. (2010) and Brennan and Owende (2010)
Botryococcus braunii	Frac et al. (2010) and Scott et al. (2010)
Nannochloropsis oculata	Mata et al. (2010) and Frac et al. (2010)
Chlamydomonas sp.	Nigam and Singh (2011) and Scott et al. (2010)
Dunaliella salina	Scott et al. (2010)
Scenedesmus sp.	Mata et al. (2010) and Sathish and Sims (2012)
Haematococcus pluvialis	Brennan and Owende (2010) and Scott et al. (2010)
Crypthecodinium cohnii	Brennan and Owende (2010)
Anabaena sp.	Brennan and Owende (2010)
Galdieria sulphuraria	Brennan and Owende (2010)
Arthrospira platensis	Brennan and Owende (2010)
Chlorococcum sp.	Brennan and Owende (2010)
Schizochytrium sp.	Frac et al. (2010)
Porphyridium cruentum	Brennan and Owende (2010)

and technological development will be needed (Bender 1999; Demirbas 2003).

There are still many technical challenges to be overcome for the large-scale production of biodiesel. In particular, genetic tools may lead to the construction of strains with desired characteristics, such as high oil contents. Nevertheless, the economic feasibility of biodiesel might be achieved progressively by combining the fuel production with high-value by-products for food and feed ingredients to hopefully meet the growing energy demand in the future (Brennan and Owende 2010; Wijffels and Barbosa 2010).

Biohydrogen

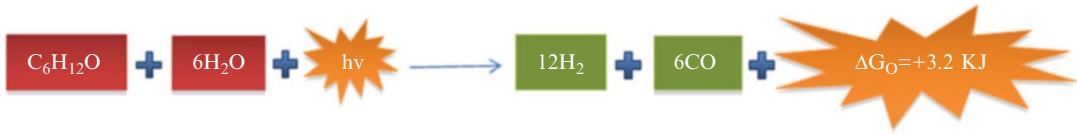
Hydrogen can serve as a significant alternate energy carrier to fossil fuel with high energy content per unit mass of any known fuel (143 GJ t^{-1}) and easily converted to electricity by fuel cells and on combustion it gives water as the only by-product. However, hydrogen is gaseous even at very low temperatures, so the storage density is an issue in any potential vehicular fuel application. Hydrogen can be produced from different methods, but biological methods of hydrogen production are preferable as they utilise CO_2, sunlight and organic wastes as substrates under moderate conditions and considered environmentally benign conversions (Redwood et al. 2009). On the other hand, chemical methods for hydrogen production are energy intensive processes requiring high temperatures (>850°C) (Kapdan and Kargi 2006).

Direct biophotolysis of H_2 production is a biological process which utilises solar energy and photosynthetic systems of algae to convert water into chemical energy.

$$2H_2O + \text{solar energy} \rightarrow 2H_2 + O_2$$

Photosystem I (PSI) and photosystem II (PSII) are used in process of photosynthesis. PSI produces reductant for CO_2, and PSII splits water to evolve O_2 or the reductant generated by photosynthesis directly transferred to hydrogenase via reduced ferredoxin ($2H^+ + 2Fd \rightarrow H_2 + 2Fd$) (Schnackenberg et al. 1996). The green algae and cyanobacteria (blue-green algae) contain hydrogenase and thus have the ability to produce H_2 (Ni et al. 2006). In these organisms, electrons are generated when PSII absorbs light energy, which is then transferred to ferredoxin. A reversible hydrogenase accepts electrons directly from the

Fig. 8.2 Production of biohydrogen via indirect photolysis method



reduced ferredoxin to generate H_2 in the presence of hydrogenase (Das et al. 2008). The reversible hydrogenase and nitrogenase are sensitive to the oxygen, which is a major barrier for sustained hydrogen evolution. In cyanobacteria (e.g., *Anabaena* strains), heterocyst provides an oxygen-free environment to the oxygen-sensitive nitrogenase that reduces molecular nitrogen into NH3 as well as protons into H_2 (Smith et al. 1992). Some green algae, for example, *Chlamydomonas reinhardtii,* deplete oxygen during oxidative respiration (Melis et al. 2000) and converts up to 22% of light energy into hydrogen energy which is equivalent to 10% solar energy conversion efficiency (Benemann 1996).

The process of indirect biophotolysis is completed in two separate stages that are joined via CO_2 fixation. CO_2 serving as an electron carrier between water splitting reaction and hydrogenase reaction. In first stage, CO_2 is fixed in the form of storage carbohydrates (starch or glycogen) followed by second stage in which carbohydrate convert to H_2 by reversible hydrogenase by both in dark- and light-driven anaerobic metabolic processes (Fig. 8.2).

The photosynthetic bacteria or non-sulphur bacteria are being used for long for H_2 production. They produce H_2 using light energy and organic acids (lactic, succinic and butyric acids or alcohols) as electron donors by mainly nitrogenase in anoxic condition. These bacteria have been found suitable for hydrogen production by using organic waste as substrate in batch processes and continuous cultures or immobilised whole cell system using different solid matrices (agar, porous glass, and polyurethane foam) (Das et al. 2008). The overall reaction is as follows:

Photofermentation has advantage over biophotolysis due to lack of PSII, which eliminates the difficulties of H_2 production by inhibitory action of oxygen. The major drawbacks of the process are low photochemical efficiencies and non-homogeneity of light distribution in bioreactor. The maximum reported production rate is 6.55 mL H_2/L h using malic acid as substrate (Tang et al. 2008).

While dark fermentation is a process in which organic substrate is converted to hydrogen by diverse group of bacteria under anaerobic conditions. The oxidation of substrate by bacteria generates electron which under anaerobic condition accepted by protons and reduced to molecular H_2 (Das et al. 2008). Although hydrogen production also reported by utilising glucose as substrate by pure cultures, but utilisation of industrial wastewater as a substrate has been drawing considerable interest in recent years due to simultaneous waste treatment and inexpensive energy generation from low-cost substrate. Dark fermentation by using mixed consortia is a complex process in which hydrolytic microorganisms

Table 8.6 Hydrogen-producing microbes

Culture	References	Culture	References
Bacillus coagulans	Fascetti and Todini (1995)	*Lactobacillus delbrueckii*	Xing et al. (2010)
C. butyricum	Wang et al. (2011)	*Pseudomonas* sp. *GZ1*	Logan (2004)
C. pasteurianum	Wang and Wan (2008)	*Rhodobacter capsulatus*	Su et al. (2010)
C. acetobutylicum	Fan et al. (2006)	*Rhodobacter* sp. *M-19*	Wang et al. (2011)
Caldicellulosiruptor	Wang et al. (2003)	*R. sphaeroides*	Sveshnikov et al. (1997)
Clostridium sp.	Ferchichi et al. (2005)	*Rhodopseudomonas*	Su et al. (2009)
C. bifermentans	Liu et al. (2011)	*R. capsulatus*	Wang et al. (2003)
E. coli HD701	Yokoyama et al. (2007)	*R. palustris*	Zhao and Yu (2008)
Enterobacter aerogenes HO-39	Yokoi et al. (2002)	*Thermoanaerobium kodakaraensis KOD1*	Idania et al. (2005)
E. cloacae IIT-BT 08	Kumar and Das (2001)	*T. cterium*	Ghirardi et al. (2000)
Enterobacter sp. *HO-39*	Wang et al. (2011)	*T. cterium*	Ghirardi et al. (2000)
Halobacterium salinarum	Zabut et al. (2006)	*Thermotoga neapolitana*	Doi et al. (2010)
Klebsiella oxytoca HP1	Federov et al. (1998)		

hydrolyze complex organic polymers to monomers which further converted to a mixture organic acids and alcohols by H_2 producing acidogenic bacteria (Pandu and Joseph 2012). Some of the hydrogen producing bacteria has been listed in Table 8.6.

Interestingly, a hybrid fermentation technology of dark fermentation and photofermentation in which light-independent bacteria and photosynthetic bacteria provide an integrated system for maximising hydrogen yield has also been used for hydrogen production (Eroglu et al. 2006). It provides maximum substrate utilisation which was thermodynamically limited in single-stage processes. It is a two-stage fermentation in which anaerobic fermentation of organic wastes produces low molecular weight intermediate (organic acids), which are then converted to hydrogen by photofermentation in photo bioreactor. The maximum yield was reported with sucrose (14.2 mol/mol) by utilising *Caldicellulosiruptor* and photosynthetic bacteria of *Rhodopseudomonas capsulata* (Wang and Wan 2008).

The main problem in the commercialisation of Biohydrogen is its high cost; therefore, novel strategies should be developed to make it more economically feasible. One of the ways to decrease the Biohydrogen production cost is to use lignocellulosic biomass (Chang et al. 2011). Several types of dedicated energy crops have been identified for biohydrogen production such as switchgrass, willow crops, hybrid poplar, alfalfa and corn stover. However, lignocellulosic biomass is usually not easily degraded by microorganisms due to their structural complexity. Pretreatment is thus necessary to lower down the lignin content, reduce the crystal structure and increase the surface area of the substrate (Xia and Sheng 2004).

A comparison of energy costs for different biological processes and the non-biological processes has been made by several investigators. The direct production of H_2 from biomass eliminates the need for electrolysis, resulting in higher system efficiencies. The cost at ~€40/million of Btu of fermentative H_2 production at 10% conversion efficiency is considered to be unattractive. However, by adopting immobilised systems, an increase in the conversion efficiency up to 28.34% has been proposed (Kumar and Das 1999, 2000). In contrast, photobiological H_2 production at 10% conversion efficiencies estimated to cost ~€10/million Btu. It is attractive when compared with the fermentative ethanol production that costs ~€31.5/million Btu at 1–30% conversion efficiencies. In addition, lower costs due to fewer requirements for pretreatment in comparison to ethanol production from biomass offer a great advantage.

However, the diversion of biomass for H_2 production by dark fermentation and/or electricity

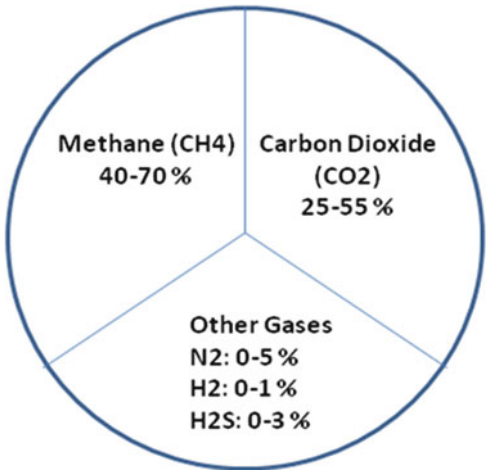


Fig. 8.3 Composition of biogas

generation would greatly depend on the technological maturity and land availability. In addition short duration, high density, fast growing and easily biodegradable dedicated energy crops would be needed. Similarly, long-term R&D efforts would be essential for the better conversion efficiency.

Biogas and Biomethane

Uncontrolled waste dumping is a serious problem of contemporary human habitation today; its controlled landfill disposal and incineration of organic wastes are not even considered optimal practices. In this context, energy recovery and recycling of organic matter and its nutrients through anaerobic digestion has been prioritised nowadays as an economical, immensely useful and greener technology. Production of biogas through anaerobic digestion of animal manure and slurries as well as of a wide range of digestible organic wastes including lignocellulosic wastes of agriculture is a process of significant environmental importance (Frigon and Guiot 2010). Anaerobic digestion converts these substrates into biogas, containing about 60% methane and 40% other gases, mainly carbon dioxide, and traces of nitrogen, hydrogen and hydrogen sulphide (Fig. 8.3). Resultant combustible gaseous product is usually termed as biogas, while biomethane is a term used to describe a gas mixture that is predominantly methane (>97%) obtained after upgrading biogas.

There are numerous benefits associated with this renewable technology. It offers a clean and particulate-free source of energy. Biogas technology is a particularly useful system in the rural economy of any developing country like India and can fulfil several end uses. The gas is useful as a fuel substitute for firewood, dung, agricultural residues, petrol, diesel and electricity, depending on the nature of the task. Moreover, the slurry that is formed after methanogenesis is superior in terms of its nutrient content as the process of methane production serves to narrow the C:N, while a fraction of the organic N is mineralised to ammonium and nitrate, the form which is immediately available to plants.

The biogas process is a natural biological process that requires cooperation between different microorganisms and groups of microorganisms to function properly. Biogas microbes consist of a large group of complex and differently acting microbe species, notable the methane-producing bacteria (Table 8.7). The whole biogas process can be divided into three steps: hydrolysis, acidification (fermentation and acetogenesis) and methane formation (Fig. 8.4). Methane and acid-producing bacteria act in a symbiotically under anaerobic digestion process. On one hand, acid-producing bacteria create an atmosphere with ideal parameters for methane-producing bacteria, while on the other hand, methane-producing microorganisms use the intermediates of the acid-producing bacteria.

There are two prominent methods of biogas production: using anaerobic digester and landfill gas.

Anaerobic digestion is a biochemical process whereby organic biomass sources are broken down by a diverse population of microorganisms in a low-oxygen environment, thus producing biogas as a natural by-product. Since the microorganisms are already present in all organic material, the process is triggered once the biomass is placed in a low-oxygen environment. Almost any organic material is a potential source of biomass feedstock to produce biogas using anaerobic

Table 8.7 Methane-producing microorganisms

Microorganism	References	Microorganism	References
Methanobrevibacter gottschalkii strain PG	Jin et al. (2011)	*Methanosphaera cuniculi, M. stadtmanae*	Garcia (1990)
Methanobrevibacter thaueri CW	Jin et al. (2011)	Methanothermus *fervidus, M. sociabilis*	Garcia (1990)
Methanobrevibacter spp.	Jin et al. (2011)	*Methanococcus aeolicus, M. delme, M. halophilus, M. jannaschii, M. maripaludis, M. thermolithotrophicus, M. vannielii, M. voltae*	Garcia (1990)
Methanobrevibacter ruminantium	Tavendale et al. (2005)	*Methanomicrobium mobile*	Garcia (1990)
Ruminococcus flavefaciens	Mao et al. (2010)	*Methanolacinia paynteri*	Garcia (1990)
Fibrobacter succinogenes	Mao et al. (2010)	*Methanospirillum hungatei*	Garcia (1990)
Methanosarcina mazei	Green et al. (2008)	*Methanogenium annulus, M. bourgense, M. cariaci*	Garcia (1990)
Methanobacterium alcaliphilum, M. bryantii, M. espanolae, M. formicicum, M. ivanovii, M. palustre, M. thermoaggregans, M. thermoalcaliphilum, M. thermoautotrophicum, M. therrnoformicicum, M. uliginosum	Garcia (1990)	*Methanosarcina mazei, Methanosarcina lacustris, Methanocorpusculum* sp., *Methanomethylovorans hollandica*	Simankova et al. (2003)
Methanobrevibacter arboriphilus, M. ruminantium, M. smithii	Garcia (1990)	*Methanosarcina barkeri*	Kadam et al. (1989)

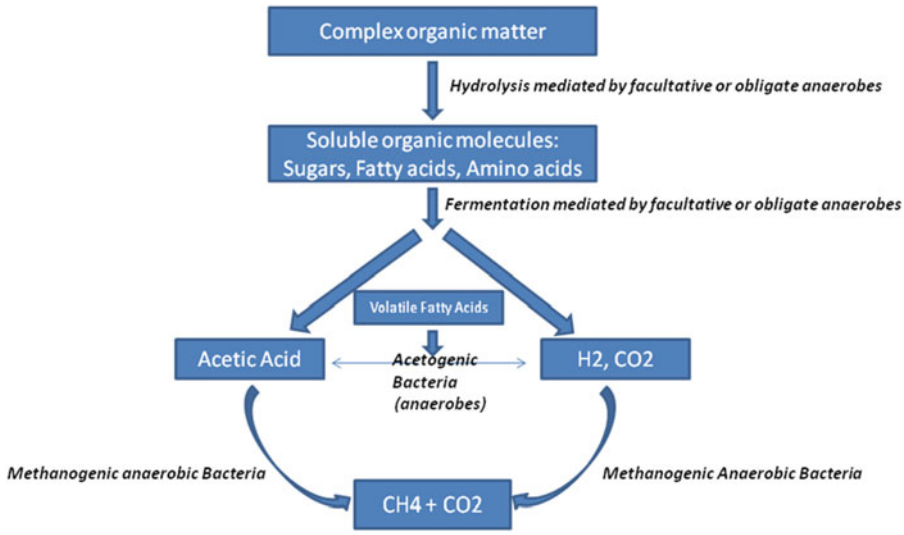


Fig. 8.4 Microbiology of biogas production during anaerobic digestion

digester (Nallathambi 1997). Sewage, manure, forestry wastes, agricultural wastes, energy crops, and industrial food processing wastes may be the most common biomass feedstocks for biogas production (Chynoweth et al. 2001). Biogas from sewage treatment plant digesters usually contains 55–65% methane, 35–45% carbon dioxide and <1% nitrogen, while biogas from other organic

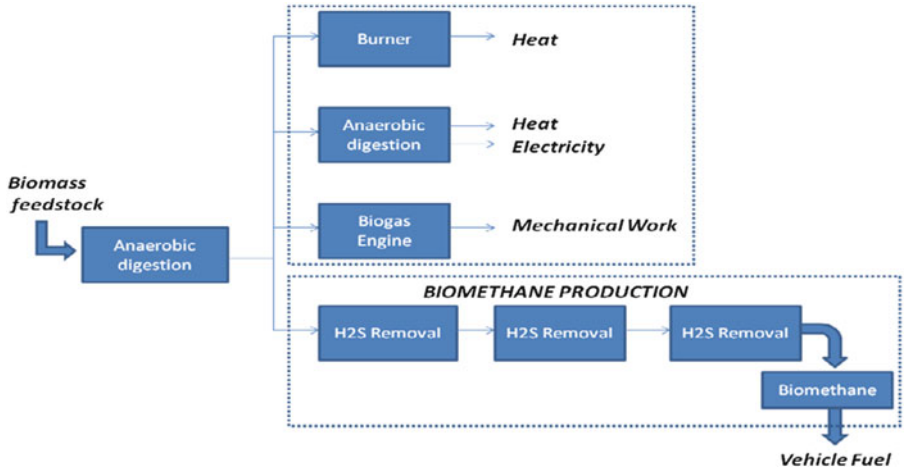


Fig. 8.5 Schematic diagram of biogas and biomethane production and their utilisation

waste digesters usually contains 60–70% methane, 30–40% carbon dioxide and <1% nitrogen (Ras et al. 2007).

Landfill disposal is a predominant method of waste management. However, landfilling is unsustainable due to its harmful effects on the environment and public health. Therefore, biodegradable municipal waste (BMW) from landfills is required to divert to be used for landfill gas production. This is prominent technology towards development of energy from waste and is functional in Europe, the USA and other countries (Raven and Gregersen 2007). Landfill gas is a water-saturated gas mixture containing about 40–60% methane, with the remainder being mostly carbon dioxide (Asgari et al. 2011). Landfill gas also contains varying amounts of nitrogen, oxygen, water vapour, sulphur and a hundreds of other contaminants. Inorganic contaminants like mercury are also known to be present in landfill gas.

Biomethane production involves upgrading, or 'cleaning-up' of raw biogas to a higher-quality gas containing primarily biomethane. As a raw gas, biogas doesn't contain the energy potential to be used for a number of applications such as gas grid injection or as a vehicle fuel (Fig. 8.5). Biogas upgrading involves removal of carbon dioxide, hydrogen sulphide, water vapour as well as trace gases. The resulting biomethane usually have a higher content of methane and a higher energy content making it essentially identical to conventional natural gas. There are number of different upgrading method which can be used to increase CH_4 concentration (Wellinger and Lindberg 1999; Ryckebosch et al. 2011). The primary steps in the biogas upgrading process are as follows:

Since the CH_4 content is directly proportional to its energy content, increasing the methane content results in higher calorific value. Membrane separation, pressure swing adsorption (PSA) and water scrubbing are some of the prominent methods used to increase CH_4 by removing CO_2 from biogas (Wellinger and Lindberg 1999). Scrubbing with water is one of the cheapest and most common techniques for this purpose. Organic physical scrubbing includes solvents such as polyethylene glycol instead of water. Membrane separation and pressure swing adsorption (PSA) are different to absorption scrubbing techniques. A membrane is used from which water, O_2 and CO_2 are able to permeate through while a very limited amount of CH_4 and nitrogen is able to pass (Wellinger and Lindberg 1999; Makaruk et al. 2010). Activated carbon and other molecular sieves can be used where the gas is fed through a series of columns in pressure, by which the CO_2 is adsorbed to column matrix and CH_4 reaches to the top of the vessel (Petersson and Wellinger 2009). H_2S is also a common contaminant present in biogas, which can be removed by in situ reduction

of H_2S within the digester vessel by adding metal ions. Removal of H_2S can be carried out via metal oxides, oxidation with air, adsorption on activated carbon and biological approach (Wellinger and Lindberg 1999). Chemical-oxidative scrubbing is also a promising technique for the removal of hydrogen sulphide from raw biogas (Miltner et al. 2012). Raw biogas is saturated with water vapour. Since water is potentially damaging to natural gas pipeline equipment and engines, it needs to be removed. The removal of water is performed via a number of different methods at varying points in the biogas upgrading process. Refrigeration, adsorption and absorption are some of the most common methods used for removing water from biogas (Wellinger and Lindberg 1999). In addition to H_2S, H_2O and CO_2, there may be other trace contaminants present in the biogas which are potentially harmful to equipment and/or people and must therefore be removed or reduced to acceptable levels.

There have also been new developments in upgrading process of biogas such as cryogenic separation which is based on the sublimation points of different gases. Compressing and cooling the gas down to different temperatures, separation of various contaminants occurs. For instance, at −25°C, sulphur dioxide, siloxanes, water and H_2S are removed; between −50 and −59°C, up to 40% of the CO_2 is removed as a liquid; and finally, the remaining CO_2 get removed in solid form when biogas cools further.

Advanced Energy Carriers

Furans

DMF is also emerging as a potential transportation fuel alternative, which have several properties such as 40% higher energy density, a boiling point 20°C higher than the ethanol and a lower solubility in water. DMF can be produced from the dehydration of HMF, a dehydration product of hexose sugars. HMF can be produced from sugars and lignocelluloses via dissolution and dehydration. Nevertheless, it is an intermediate, having a boiling point too high to be used directly as a transportation fuel. Thus, it should be derivatised to other compounds, such as furan derivatives, particularly dimethylfuran (DMF), which is a suitable compound for gasoline-range fuel (James et al. 2010). HMF esters are considered as relatively stable (Gruter et al. 2009) and, consequently, can be used as such or further derivatised.

Several noble metal catalysts were applied in the hydrogenolysis of HMF. Since these catalysts are degraded by acids, a neutralisation step is needed. Thus, a two-step method for the production of DMF from glucose has been proposed (Chidambaram and Bell 2010). Initially, 12-molybdophosphoric acid ($H_3PMo_{12}O_{40}$) was used as the catalyst in a $[C_2MIM][Cl]$ ionic liquid-acetonitrile for the dehydration of glucose to produce HMF. As the second step, hydrogenation and hydrogenolysis of HMF were carried out over Pd/C catalysts. However, before second step, the temperature of the reaction mixture was cooled down to 50°C and neutralised (Maki-Arvela et al. 2012).

Methanol

Similar to other conventional biofuels, biologically derived methanol has also garnered tremendous interest. Although traditionally methanol is produced via a non-sustainable and cost-intensive chemical process involving catalytic steam reforming of natural gas, it is possible to produce this fuel in an environmentally benign manner using biomass resources (Demirbas 2008; Zinoviev et al. 2010). The advantages of using methanol as a fuel were realised long time ago and were introduced into the fuel economy (Reed and Lerner 1973). Thereafter, research and development on methanol production gained momentum applying various strategies. Before modern production technologies were developed, methanol was obtained from wood as a coproduct of charcoal production and, for this reason, it was commonly known as wood alcohol. It is produced from hydrogen and mixture of oxides of carbon by means of the catalytic reaction. Biosynthesis gas (bio-syngas) is a gas rich in CO and H_2 obtained by gasification of biomass, which is available in renewable basis (Fig. 8.6).

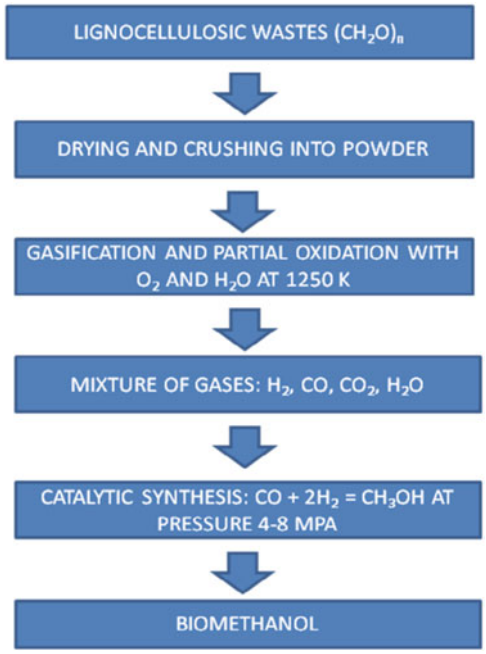


Fig. 8.6 Biomethanol production from lignocellulosic waste

Methanol is currently manufactured worldwide by conversion or derived from bio-syngas, natural gas, refinery off-gas, etc. (Demirbas and Gulu 1998.), according to following reaction:

$$2H_2 + CO = CH_3OH$$

Biomethanol can be an indispensable fuel with multiple applications. First and foremost, it can be used as a motor fuel in conventional engines, in its pure form or as a blend with gasoline, with an excellent emission profile. It is also possible to directly covert methanol to gasoline (Stelmachowski and Nowicki 2003; Demirbas 2008; Zinoviev et al. 2010). Second, it can be converted to MTBE (methyl tert-butyl ether), an additive to gasoline. While MTBE is a formidable fuel additive and enhancer, its production process involves using isobutylene, a product derived from fossil fuels. Third, it can be dehydrated to produce DME (dimethyl ether), a suitable replacement for natural gas. Lastly, it can be used as a raw material in the production of biodiesels (as FAMEs, fatty acid methyl esters) (Demirbas 2008; Zinoviev et al. 2010).

A variety of catalysts are capable of causing the conversion, including reduced NiO-based preparations, reduced Cu/ZnO shift preparations, Cu/SiO_2 and Pd/SiO_2 and Pd/ZnO (Demirbas and Demirbas 2007). Since the methanol production is a costly process, waste biomass should be considered for as potential cost-effective substrate. Biomass and coal have been considered as a potential fuel for gasification and eventually for syngas production and methanol synthesis. Adding sufficient hydrogen to the synthesis gas to convert all of the biomass into methanol can double the methanol produced from the same biomass base (Phillips et al. 1990). The natural gas is converted to methanol in a conventional steam reforming/water gas shift reaction followed by high-pressure catalytic methanol synthesis:

$$CH_4 + H_2O = CO + 3H_2$$
$$CO + H_2O = CO_2 + H_2$$
$$\text{Finally, } CO + 2H_2 = CH_3OH$$

However, quite recently, efforts are being made to produces biomethanol from crude glycerine, a renewable by-product of biodiesel synthesis.

Butanol

The four-carbon alcohols, 'butanol', are the longest chain alcohols found as natural major end-products of microbial fermentation. With the exception of a relatively small proportion of butanol produced by fermentation in China and Brazil (Ni and Sun 2009), these alcohols are currently petrochemically synthesised. The annual production of butanol has been estimated to be 2.8 million tons (2006) with continuous increase in demand and capacity. Butanol has many characteristics that make it a better fuel than ethanol. Butanol has the following advantages over ethanol: (a) it has 25% more Btu per gallon, (b) it is less evaporative/explosive with a Reid vapour pressure (RVP) 7.5 times lower than ethanol, (c) it is safer than ethanol because of its higher flash point and lower vapour pressure, (d) it has a higher octane rating, and (e) it is more miscible with

Table 8.8 List of microbes producing butanol and isopropanol

Alcohols	Microorganism	References
Butanol	*Clostridium beijerinckii*	Survase et al. (2011)
	Metabolic engineered *E. coli*	Atsumi et al. (2008)
	Clostridium beijerinckii	Formanek et al. (1997)
	Clostridium acetobutylicum	Ennis et al. (1987) and Qureshi and Maddoxm (1991)
	Synechococcus elongatus	Lan and Liao (2012)
	Bacillus sp.	Wright et al. (1991)
2-Propanol	*Thermoanaerobacter ethanolicus*	Burdette et al. (1996)
	Thermoanaerobium brockii	Keinan et al. (1986)
	Clostridium beijerinckii	Chen (1995) and Jojima et al. (2008)
	Clostridium isopropylicum	Inokuma et al. (2010)
	Clostridium ljungdahlii	Imkamp and Muller (2007) and Ramachandriya et al. (2011)
	Clostridium autoethanogenum	Imkamp and Muller (2007) and Ramachandriya et al. (2011)
	Butyribacterium methylotrophicum	Imkamp and Muller (2007) and Ramachandriya et al. (2011)
	Clostridium ragsdalei	Imkamp and Muller (2007) and Ramachandriya et al. (2011)
	Clostridium carboxidivorans	Imkamp and Muller (2007) and Ramachandriya et al. (2011)
	Escherichia coli	Hanai et al. (2007) and Atsumi and Liao (2008)

gasoline and diesel fuel but less miscible with water (Ramey and Yang 2004). It is produced via acetone-butanol-ethanol fermentation (ABE fermentation) with the strict anaerobic bacterium, *Clostridium acetobutylicum* and *C. beijerinckii* (Qureshi and Blaschek 2001; Ramey and Yang 2004; Survase et al. 2011). However, with a few exceptions, anaerobic fermentation processes for production of fuels and chemicals, including ABE fermentation, usually suffer from a number of serious limitations including low yields, low productivity and low final product concentrations. Efforts are being made to make fermentation route competitive with petroleum-based solvent synthesis because the limitations have also been overcome (Chauvatcharin et al. 1998). However, petrochemical route of butanol production is still dominated over ABE fermentation. Few butanol-producing microbes are listed in Table 8.7.

Isopropanol

Isopropanol is one of the simplest secondary alcohols which are produced by microbes. Several species of *Clostridium* have been evaluated for isopropanol production, including *Clostridium beijerinckii* and *Clostridium isopropylicum* IAM 19239. However, the obtained isopropanol titres of these strains were very low, because clostridia produce isopropanol together with butanol (Table 8.8). Accumulation of isopropanol drastically reduced production yields. In this context, Inokuma et al. (2010) have recently applied a gas-stripping recovery method into the fed-batch culture system. Applying this method, they have successfully obtained production of 2,378 mM (143 g/L) of isopropanol from a recombinant clone of *E. coli* after 240 h with a yield of 67.4% (mol/mol) which is very close to the theoretical maximum yield (73.2% (mol isopropanol/mol glucose)).

Chemically, the isopropanol is primarily produced by combining water and propene in a hydration reaction at industrial level (Lee et al. 2003). There are two routes for the hydration process: indirect hydration via the sulphuric acid process and direct hydration. In the indirect process, propene reacts with sulphuric acid to form a mixture of sulphate esters. Subsequent hydrolysis of these esters by steam produces isopropyl alcohol, which is purified after distillation. In direct hydration, propene and water react at high pressures in the presence of solid or supported acidic catalysts

either in gas or liquid phases. Higher purity propylene (>90%) tends to be required for this type of process.

Thermochemical conversion of lignocellulosic feedstock into various advanced primary and secondary alcohol is an emerging technology which is known as producer gas fermentation (also known as synthesis gas or bio-syngas fermentation). It involves the use of acidogenic biological catalysts (such as *Clostridium ljungdahlii, Clostridium autoethanogenum, Butyribacterium methylotrophicum, 'Clostridium ragsdalei'* and *Clostridium carboxidivorans* P7, Table 8.8) which have an autotrophic mechanism of converting syngas components (CO, CO_2 and H_2 primarily) into solvents such as ethanol and butanol and commodity chemicals such as acetic acid and butyric acid (Imkamp and Muller 2007; Ramachandriya et al. 2011).

Dimethyl Ether (DME)

DME (CH_3OCH_3) is the simplest ether. It represents a new renewable fuel that still needs further exploration. It does not occur naturally in petroleum, hence is produced synthetically. The physical properties of DME are similar to LPG, as it can be transported and stored as a liquid at low temperature. DME is a clean fuel and contains no S or N compounds. Its energy content is ~65% that of CH_4. When used as a replacement for diesel fuel, DME has a high cetane value, which makes it more suitable for application in CI ICE (compressed ignition internal combustion engine) rather than in SI ICE (spark ignited internal combustion engine) (Semelsberger et al. 2006; Swaina et al. 2011).

Originally DME has been manufactured via methanol dehydration, but more recently the direct DME production from syngas (mixture of CO and H_2). The direct production route involves one process instead of two processes – methanol synthesis and methanol dehydration and appears to be more energy and cost efficient. The cost of producing DME from methanol is influenced by price and availability, as methanol itself is an expensive chemical feedstock. In contrast, producing DME directly from syngas has many economic and technical advantages over methanol dehydration. Thermodynamically, DME production from syngas is more favourable than from methanol, and thus, in principle, the costs for DME production from syngas should be lower, provided a suitable catalyst can be found. Considering the exothermic characteristic of DME synthesis and the scale of production, we explored the feasibility of utilising a micro-channel reactor for DME synthesis, in conjunction with a combined methanol synthesis/dehydration catalyst system (Hu CK 2005; Hu J 2005).

Dimethyl ether can address energy security, energy conservation, environmental concerns and the pragmatic realisation of depleting petroleum reserves as an alternative fuel. Besides it can be exploited as a nontoxic, noncorrosive, environmentally benign, produced from domestic resources.

Pyrolysis Oil

Biomass can be processed in a liquid media (typically water) under pressure and at temperatures between 300 and 400°C. The reaction yields oils and residual solids that have a low water content and a lower oxygen content than oils from fast pyrolysis (www.nabcprojects.org). Upgrading of the so-called bio-crude is similar to that of pyrolysis oil. Pyrolysis oil can be produced by fast pyrolysis, a process involving rapidly heating the biomass to temperatures between 400 and 600°C, followed by rapid cooling. Through this process, thermally unstable biomass compounds are converted to a liquid product. The obtained pyrolysis oil is more suitable for long-distance transport than for instance straw or wood-chips. As a by-product, bio-char is produced that can be used as solid fuel, or applied on land as a measure of carbon sequestration and soil fertilisation. The oil can be processed in ways similar to crude oil, and several research efforts are currently undertaken to upgrade pyrolysis oil to advanced biofuels (EBTP 2010).

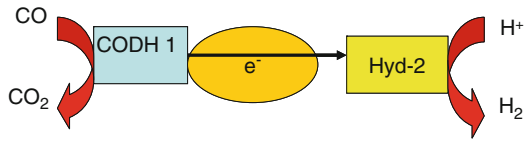


Fig. 8.7 Hydrogenase-mediated hydrogen production

Microbial Batteries

Microbial fuel cells (MFCs) open up new horizons for the sustainable energy production from biodegradable organic matters. The microbial batteries convert energy of bio-convertible substrate into electricity. This can be achieved when bacteria switch from the natural electron acceptor to an insoluble acceptor, such as the fuel cell's anode. MFCs have operational and functional advantages over the technologies currently used for generating energy from organic matter such as high conversion efficiency, operation at ambient temperature, does not require gas treatment, do not need energy input for aeration and have potential for widespread application.

Many organisms identified in microbial batteries possess hydrogenase enzyme, for example, *Enterobacter*, *Bacillus, Clostridium, Bacteroidetes*, *Actinobacteria* and 11 novel phylotypes closely related to *Ethanoligenens harbinense*, *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* were also there (Xing et al. 2008). Hydrogenases could be directly involved in electron transfer towards electrodes. Hydrogenase is thought to become active in order to excrete excess reducing power under specific conditions, such as anaerobic conditions. Although, hydrogenase enzyme is purely responsible for this hydrogen production, which catalyses the following reaction:

$$2H^+ + 2X_{\text{reduced}} \leftrightarrow 6H_2 + 2X_{\text{oxidised}}$$

as illustrated in (Fig. 8.7).

Bacteria can use soluble components that physically transport the electron from an (intra) cellular compound, which becomes oxidised, to the electrode surface. In many studies, redox mediators were added to the reactor, which often seemed to be essential. However, bacteria can also produce redox mediators themselves in two ways: through the production of organic, reversibly reducible compounds (secondary metabolites) and through the generation of oxidisable metabolites (primary metabolites).

Conclusion

The potential to use available residues from the agricultural and forestry sector to produce biofuels underscores the need for technology development. The assessment of sustainable biomass potential and the evaluation of benefits of biofuels are important key factors for increasing rural energy access. Moreover, the investment to help build capacities in the field for feedstock supply and handling can create favourable conditions to establishing a biofuel industry.

References

Abbi M, Kuhad RC, Singh A (1996a) Bioconversion of pentose sugars to ethanol by free and immobilized cells of *Candida shehatae* (NCL-3501): fermentation behavior. Process Biochem 31:555–560

Abbi M, Kuhad RC, Singh A (1996b) Fermentation of xylose and rice straw hydrolysate to ethanol by *Candida shehatae* NCL-3501. J Ind Microbiol 17:20–23

Abdenifar S, Karimi K, Khanahmadi M, Taherzadeh MJ (2009) Ethanol production by *Mucor indicus* and *Rhizopus oryzae* from rice straw by separate hydrolysis and fermentation. Biomass Bioenergy 33:828–833

Aden A, Ruth M, Ibsen K, Jechura J, Neeves K, Sheehan J, Wallace B, Montague L, Slayton A, Lukas J (2002) Lignocellulosic biomass to ethanol process design and economics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis for corn stover. National Renewable Energy Laboratory Technical Report. NREL/TP-510-32438

Aguilera F, Peinado RA, Millan C, Ortega JM, Mauricio M (2006) Relationship between ethanol tolerance, H+-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. Int J Food Microbiol 110:34–42

Alfani A, Gallifuoco F, Saporosi A, Cantarella M (2000) Comparison of SHF and SSF process for the bioconversion of steam-exploded wheat straw. J Ind Microbiol Biotechnol 25:84–192

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 101:4851–4861

Asgari MJ, Safavi K, Mortazaeinezahad F (2011) Landfill biogas production process. In: International conference on food engineering and biotechnology. IPCBEE, vol 9. IACSIT Press, Singapore. http://www.ipcbee.com/vol9/40-B10035.pdf

Atsumi S, Liao J (2008) Metabolic engineering for advanced biofuels production from *Escherichia coli*. Curr Opin Biotechnol 19:414–419

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Katherine JY, Chou-Hanai T, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 10:305–311

Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I (2004) Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SSF) with *Kluyveromyces marxianus* CECT 10875. Process Biochem 39: 1843–1848

Belloch C, Orlic S, Barrio E, Querol A (2008) Fermentative stress adaptation of hybrids within the *Saccharomyces* sensu stricto complex. Int J Food Microbiol 122:188–195

Bender M (1999) Economic feasibility review for community-scale farmer cooperatives for biodiesel. Bioresour Technol 70:81–87

Benemann JR (1996) Hydrogen biotechnology: progress and prospects. Nat Biotechnol 14:1101–1103

EBTP (European Biofuels Technology Platform) (2010) Biofuel production. www.biofuelstp.eu/fuelproduction.html

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382:769–781

Bothast RJ, Saha BC, Flosenzier VA, Ingram LO (1994) Fermentation of L-arabinose, D-xylose, and D-glucose by ethanologenic recombinant *Klebsiella oxytoca* strain P2. Biotechnol Lett 16:401–406

Brennan L, Owende P (2010) Biofuels from microalgae – a review of technologies for production, processing and extractions of biofuels and co-products. Renew Sustain Energy Rev 14:557–577

Burdette DS, Vieille C, Zeikus JG (1996) Cloning and expression of the gene encoding the *Thermoanaerobacter ethanolicus* 39E secondary-alcohol dehydrogenase and biochemical characterization of the enzyme. Biochem J 316:115–122

Carraretto C, Macor A, Mirandola A, Stoppato A, Tonon S (2004) Biodiesel as alternative fuel: experimental analysis and energetic evaluations. Energy 29:2195–2211

Chaillou S, Pouwels PH, Postma PW (1999) Transport of D-xylose in *Lactobacillus pentosus, Lactobacillus casei,* and *Lactobacillus plantarum:* evidence for a mechanism of facilitated diffusion via the phosphoenolpyruvate: mannose phosphotransferase system. J Bacteriol 181:4768–4773

Chandel AK, Kapoor RK, Singh A, Kuhad RC (2007) Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresour Technol 98:1947–1950

Chauvatcharin S, Siripatana C, Seki T, Takagi M, Yoshida T (1998) Metabolism analysis and on-line physiological state diagnosis of acetone-butanol fermentation. Biotechnol Bioeng 58:561–571

Chen J (1995) Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. FEMS Microbiol Rev 17:263–273

Chen M, Xia L, Xue P (2007) Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. Int Biodeter Biodegr 59:85–89

Cheng C, Chung Y, Lee S, Lee DJ, Lin CY, Chang SJ (2011) Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 102:8514–8523

Chidambaram M, Bell AT (2010) A two-step approach for the catalytic conversion of glucose to 2, 5-dimethylfuran in ionic liquids. Green Chem 12(7): 1253–1262

Chou Y-C, Zhang M, Mohagheghi A, Evans K, Finkelstein M (1997) Construction and evaluation of a xylose/arabinose fermenting strain of *Zymomonas mobilis*. Abstracts in 19th symposium on biotechnology for fuels and chemicals, Colorado, Springs, 4–8 May

Chynoweth DP, Owens JM, Legrand R (2001) Renewable methane from anaerobic digestion of biomass. Renew Energy 22:1–8

Das D, Kanna N, Veziroglu TN (2008) Recent developments in biological hydrogen production processes. Chem Ind Chem Eng Q 14:57–67

Demirbas A (2003) Biodiesel fuels from vegetable oils via catalytic and non-catalytic supercritical alcohol transesterifications and other methods: a survey. Energy Convers Manage 44:2093–2109

Demirbas A (2006) Biodiesel production via non-catalytic SCF method and biodiesel fuel characteristics. Energy Convers Manage 47:2271–2282

Demirbas A (2007) Importance of biodiesel as transportation fuel. Energy Policy 35:4661–4670

Demirbas A (2008) Biomethanol production from organic waste materials. Energy Source A 30:565–572

Demirbas A, Demirbas MF (2011) Importance of algae oil as a source of biodiesel. Energy Convers Manage 52:163–170

Demirbas A, Gulu D (1998) Acetic acid, methanol and acetone from lignocellulosics by pyrolysis. Energy Educ Sci Technol 1:111–115

Deshpande V, Keskar S, Mishra C, Rao M (1986) Direct conversion of cellulose/hemicellulose to ethanol by *Neurospora crassa*. Enzyme Microb Technol 8:149–152

Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266

Dien BS, Nichols NN, O'Bryan PJ, Bothast RJ (2000) Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl Biochem Biotechnol 84:181–196

Doi T, Matsumoto H, Abe J, Morita S (2010) Application of rice rhizosphere microflora for hydrogen production from apple pomace. Int J Hydrogen Energy 35:7369–7376

EIA (2009) Energy Information Administration, Assumptions to the annual energy outlook 2009, DOE/EIA-0554. http://www.eia.doe.gov/oiaf/aeo/assumption, www.eia.doe.gov/oiaf/aeo/assumption

El Kanouni A, Zerdani I, Zaafa S, Znassni M, Loutfi M, Boudouma M (1998) The improvement of glucose/xylose fermentation by *Clostridium acetobutylicum* using calcium carbonate. World J Microbiol Biotechnol 14:431–435

Elbahloul Y, Steinbohel A (2010) Pilot-scale production of fatty acid ethyl esters by an engineered Escherichia coli strain harboring the p(Microdiesel) plasmid. Appl Environ Microbiol 76:4560–4565

Eliasson A, Christensson C, Wahlbom CF, Hahn-Hägerdal B (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. Appl Environ Microbiol 66:3381–3386

Ennis BM, Qureshi N, Maddox IS (1987) Inline toxic product removal during solvent production by continuous fermentation using immobilized *Clostridium acetobutylicum*. Enzyme Microb Technol 9:672–675

Eroglu E, Eroglu I, Gunduz U, Turker L, Yucel M (2006) Biological hydrogen production from olive mill wastewater with two stage processes. Int J Hydrogen Energy 31:1527–1535

Fan YT, Zhang YH, Zhang SF, Hou HW, Ren BZ (2006) Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresour Technol 97:500–505

Fascetti E, Todini O (1995) *Rhodobacter sphaeroides* RV cultivation and hydrogen production in a one- and two-stage chemostat. Appl Microbiol Biotechnol 22:300–305

Federov AS, Tsygankov AA, Rao KK, Hall DO (1998) Hydrogen photoproduction by *Rhodobacter sphaeroides* immobilized on polyurethane foam. Biotechnol Lett 20:1007–1009

Ferchichi M, Crabbe E, Gwang GH, Hintz W, Almadidy A (2005) Influence of initial pH on hydrogen production from cheese whey. J Biotechnol 120:402–409

Formanek J, Mackie R, Blaschek HP (1997) Enhanced butanol production by *Clostridium beijerinckii* BA101 grown in semidefined P2 medium containing 6 percent maltodextrin or glucose. Appl Environ Microbiol 63:2306–2310

Frac M, Jezierska-Tys S, Tys J (2010) Microalgae for biofuels production and environmental applications: a review. Afr J Biotechnol 9:9227–9236

Frigon JC, Guiot SR (2010) Biomethane production from starch and lignocellulosic crops: a comparative review. Biofuels Bioprod Biorefin 4(4):447–458

Garcia JL (1990) Taxonomy and ecology of methanogens. FEMS Microbiol Rev 87:297–308

Gerhard K (2010) Biodiesel and renewable diesel: a comparison. Prog Energy Combust Sci 36:364–373

Ghirardi ML, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A (2000) Microalgae: a green source of renewable hydrogen. TIBTECH 18:506–511

Goering E, Schwab W, Daugherty J, Pryde H, Heakin J (1982) Fuel properties of eleven vegetable oils. Trans ASAE 25:1472–1483

Gruter G, Manzer LE, De Sousa Dias ASV, Dautzenberg F, Purmova J (2009) Hydroxymethyl furfural ethers and esters prepared in ionic liquids. WO2009/030512

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresour Technol 100:1214–1220

Gupta R, Khasa YP, Kuhad RC (2011) Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. Carbohydr Polym 84:1103–1109

Hahn-Hägerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF (2007) Towards industrial pentose fermenting yeast strains. Appl Microbiol Biotechnol 74:937–953

Hamelinck CN (2004) Outlook for advanced biofuels. PhD thesis, Department of Science, Technology and Society and the Copernicus Institute for Sustainable Development and Innovation of Utrecht University

Hanai T, Atsumi S, Liao J (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol 73:7814–7820

Herrero A, Gomez RF (1980) Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. Appl Environ Microbiol 40:571–577

Himmel ME, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807

Ho NWY, Chen Z, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859

Hu CK, Bai FW, An LJ (2005) Effect of flocculence of a self-flocculating yeast on its tolerance to ethanol and the mechanism. Chin J Biotechnol 21:123–128

Hu J, Wang YC, Chunshe E, Douglas CS, Don J, White JF (2005) Conversion of biomass syngas to DME using a microchannel reactor. Ind Eng Chem Res 44:1722–1727

Idania VV, Richard S, Derek R, Noemi RS, Hector MPV (2005) Hydrogen generation via anaerobic fermentation of paper mill wastes. Bioresour Technol 96:1907–1913

Imkamp F, Muller V (2007) Acetogenic bacteria. Encyclopedia of life sciences. Wiley, Malden

Ingram LO, Aldrich HC, Borges AC, Causey TB, Martinez A, Morales F, Saleh A, Underwood SA, Yomano IP, York SW, Zaldivar J, Zhou S (1999) Enteric bacterial catalysts for fuel ethanol production. Biotechnol Prog 15:855–866

Inokuma K, Liao JC, Okamoto M, Hanai T (2010) Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. J Biosci Bioeng 110:696–701

James OO, Maity S, Usman LA et al (2010) Towards the conversion of carbohydrate biomass feedstocks to biofuels via hydroxylmethylfurfural. Energy Environ Sci 3:1833–1852

Jeffries TW (2006) Engineering yeasts for xylose metabolism. Curr Opin Biotechnol 17:320–326

Jeffries TW, Jin Y-S (2004) Ethanol and thermo-tolerance in the bioconversion of xylose by yeasts. Adv Appl Microbiol 47:221–266

Jeppsson M, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2002) Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. Appl Environ Microbiol 68:1604–1609

Jeppsson M, Träff K, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2003) Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. FEMS Yeast Res 3:167–175

Jeppsson M, Bengtsson O, Franke K, Lee H, Hahn-Hägerdal B, Gorwa-Grauslund MF (2006) The expression of a *Pichia stipitis* xylose reductase mutant with higher Km for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 93:665–673

Jin W, Cheng Y-F, Mao S-Y, Zhu W-Y (2011) Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. Bioresour Technol 102:7925–7931

Johansson B, Christensson C, Hobley T, Hahn-Hägerdal B (2001) Xylulokinase overexpression in two strains of *Saccharomyces cerevisiae* also expressing xylose reductase and xylitol dehydrogenase and its effect on fermentation of xylose and lignocellulosic hydrolysate. Appl Environ Microbiol 67:4249–4255

Jojima T, Inui M, Yukawa H (2008) Production of isopropanol by metabolically engineered *Escherichia coli*. Appl Environ Microbiol 77:1219–1224

Kadam PC, Godbole SH, Renade DR (1989) Isolation of methanogens from Arbian sea sediments and their salt tolerance. FEMS Microbiol Ecol 62:434

Kalscheuer R, Stoting T, Steinbohel A (2006) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529–2536

Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. Enzyme Microb Technol 38:569–582

Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF (2005) Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. Yeast 22:359–368

Karhumaa K, Fromanger R, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 73:1039–1046

Keinan E, Hafeli E, Seth K, Lamed R (1986) Thermostable enzymes in organic synthesis. 2. Asymmetric reduction of ketones with alcohol dehydrogenase from *Thermoanaerobium brockii*. J Am Chem Soc 108:162–169

Kuhad RC, Gupta R, Khasa YP, Singh A (2010a) Bioethanol production from *Lantana camara* (Red sage): pretreatment, saccharification and fermentation. Bioresour Technol 101:8348–8354

Kuhad RC, Mehta G, Gupta R, Sharma KK (2010b) Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. Biomass Bioenergy 34:1189–1194

Kuhad RC, Gupta R, Khasa YP (2011) Bioethanol production from lignocellulosics: an overview. In: Lal B, Sharma PM (eds) Wealth from waste, 3rd edn. TERI Press, New Delhi

Kuijvenhoven J (2006) Process design for an integrated lignocellulose to bioethanol production plant presentation at Netherlands Process Technology Symposium, 25 October 2006

Kumar N, Das D (1999) Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. Process Biochem 35:589–594

Kumar N, Das D (2000) Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. Proc Biochem 35:589–593

Kumar N, Das D (2001) Continuous hydrogen production by immobilized *Enterobacter cloacae* IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme Microb Technol 29:280–287

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729

Kuyper M, Winkler AA, Dijken JPV, Pronk JT (2004) Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle. FEMS Yeast Res 4:655–664

Kuyper M, Hartog MM, Toirkens MJ, Almering MJ, Winkler AA, Dijken JPV (2005) Metabolic engineering of a xylose–isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res 5:399–409

Lee WG, Lee JS, Shin CS, Park SC, Chang HN, Chang YK (1999) Ethanol production using concentrated oak wood hydrolysates and methods to detoxify. Appl Biochem Biotechnol 78:547–559

Lee GSJ, McCain JH, Bhasin MM (2003) Synthetic organic chemicals. In: Kent JA (ed) Riegel's handbook of industrial chemistry, 10th edn. Kluwer Academic/Plenum Publishers, New York, pp 851–854

Liu E, Hu Y (2010) Construction of a xylose-fermenting *Saccharomyces cerevisiae* strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. Biochem Eng J 48:204–210

Liu IC, Whang LM, Ren WJ, Lin PY (2011) The effect of pH on the production of biohydrogen by *Clostridia*: thermodynamic and metabolic considerations. Int J Hydrogen Energy 36:439–449

Logan BE (2004) Feature article: biologically extracting energy from wastewater: biohydrogen production and microbial fuel cells. Environ Sci Technol 38:160A–167A

Londo HM, Lensink SM, Deurwaarder EP, Wakker A, de Wit MP, Junginger HM, Könighofer K, Jungmeier G (2008) Biofuels development in the EU27+ until 2030: full-chain cost assessment and implications of policy options REFUEL project, WP4 final report

Lovett JC, Hards S, Clancya J, Snell C (2011) Multiple objectives in biofuels sustainability policy. Energy Environ Sci 4:261–268

Madhavan A, Tamalampudi S, Ushida K, Kanai D, Katahira S, Srivastava A, Fukuda H, Bisaria VS, Kondo A (2009) Xylose isomerase from polycentric fungus *Orpinomyces:* gene sequencing, cloning and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol. Appl Microbiol Biotechnol 82:1067–1078

Makaruk A, Miltner M, Harasek M (2010) Membrane biogas upgrading processes for the production of natural gas substitute. Sep Purif Technol 74(2010):83–92

Maki-Arvela P, Salminen E, Riittonen T, Virtanen P, Kumar N, Mikkola J (2012) The challenge of efficient synthesis of biofuels from lignocellulose for future renewable transportation fuels. Int J Chem Eng. doi:10.1155/2012/674761

Mamma D, Christakopoulos P, Koullas D, Kekos D, Macris BJ, Koukios E (1995) An alternative approach to the bioconversion of sweet sorghum carbohydrates to ethanol. Biomass Bioenergy 8:99–103

Mao H-L, Wang J-K, Zhou Y-Y, Liu J-X (2010) Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. Livest Sci 129:56–62

Marchetti JM, Miguel VU, Errazu AF (2007) Possible methods for biodiesel production. Renew Sustain Energy Rev 11:1300–1311

Margeot A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F (2009) New improvements for lignocellulosic ethanol. Curr Opin Biotechnol 20:372–380

Mata TM, Martins AA, Nidia S (2010) Caetano, microalgae for biodiesel production and other applications: a review. Renew Sust Energy Rev 14:217–232

Matsushika A, Inoue H, Watanabe S, Kodaki T, Makino K, Sawayama S (2008) Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, NADP+-dependent xylitol dehydrogenase and xylulokinase. J Biosci Bioeng 105:296–299

Melis A, Zhang LP, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 122(1):127–136

Millati R, Edebo L, Taherzadeh MJ (2005) Performance of *Rhizopus, Rhizomucor and Mucor* in ethanol production from glucose, xylose and wood hydrolysates. Enzyme Microb Technol 36:294–300

Miltner M, Makaruk A, Krischan J, Harasek M (2012) Chemical-oxidative scrubbing for the removal of hydrogen sulphide from raw biogas: potentials and economics. Water Sci Technol 66(6):1354–1360

Mosier NS, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch MR (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686

Mountfort DO, Rhodes LL (1991) Anaerobic Growth and fermentation characteristics of *Paecilomyces lilacinus* isolated from M*ullet* gut. Appl Environ Microbiol 57:1963–1968

Nagel N, Lemke P (1990) Production of methyl fuel from microalgae. Appl Biochem Biotechnol 24:355–361

Nallathambi GV (1997) Anaerobic digestion of biomass for methane production: a review. Biomass Bioenergy 13:83–114

Ni Y, Sun Z (2009) Recent progress on industrial fermentative production of acetone–butanol–ethanol by Clostridium acetobutylicum in China. Appl Microbiol Biotechnol 83:415–423

Ni M, Leung DYC, Leung MKH, Sumathy K (2006) An overview of hydrogen production from biomass. Fuel Process Technol 87:461–472

Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energy Combust Sci 37:52–68

Ohgren K, Bura R, Lesnicki G, Saddler J, Zacchi G (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem 42:834–839

Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Genetic improvement of Escherichia coli for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57:893–900

Palmqvist E, Hahn-Hägerdal B (2000a) Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour Technol 74:17–24

Palmqvist E, Hahn-Hägerdal B (2000b) Fermentation of lignocellulosic hydrolysates. II: inhibition and detoxification. Bioresour Technol 74:25–33

Pandu K, Joseph S (2012) Comparisons and limitations of biohydrogen production processes: a review. Int J Adv Eng Technol 2:342–356

Patel GB (1984) Ethanol production during o-xylose, L-arabinose, and D-ribose fermentation by *Bacteroides polypragmatus*. Appl Microbiol Biotechnol 20:111–117

Petersson A, Wellinger A (2009) Biogas upgrading technologies – developments and innovations. http://www.ieabiogas.net/Dokumente/upgrading_rz_low_final.pdf

Phillips VD, Kinoshita CM, Neill DR, Takashi PK (1990) Thermochemical production of methanol from biomass in Hawaii. Appl Energy 35:167–175

Pryor RW, Hanna MA, Schinstock JL, Bashford LL (1982) Soybean oil fuel in a small diesel engine. Trans ASAE 26:333–338

Qureshi N, Blaschek HP (2001) Recovery of butanol from fermentation broth by gas stripping. Renew Energy 22:557–564

Qureshi N, Maddoxm IS (1991) Integration of continuous production and recovery of solvents from whey permeate: use of immobilized cells of *Clostridium acetobutylicum* in a fluidized bed reactor coupled with gas stripping. Bioprocess Eng 6:63–69

Ramachandriya KD, Wilkins MR, Delorme MJM, Zhu X, Kundiyana DK, Atiyeh HK, Huhnke RL (2011) Reduction of acetone to isopropanol using producer gas fermenting microbes. Biotechnol Bioeng 108:2330–2338

Ramey D, Yang ST (2004) Production of butyric acid and butanol from biomass. U.S. Department of Energy Morgantown, WV. http://www.afdc.energy.gov/pdfs/843183.pdf

Ras S, Veijanen A, Rintala J (2007) Trace compounds of biogas from different biogas production plants. J Energy 32:1375–1380

Raven RPJM, Gregersen KH (2007) Biogas plants in Denmark: successes and setbacks. Renew Sustain Energy Rev 11:116–132

Redwood MD, Paterson-Beedle M, Macaskie LE (2009) Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy. Rev Environ Sci Biotechnol 8:149–185

Reed TB, Lerner RM (1973) Methanol: a versatile fuel for immediate use. Science 182:1299–1304

Rocha MVP, Rodrigues TH, de Macedo GR, Gonçalves LR (2009) Enzymatic hydrolysis and fermentation of pretreated cashew apple bagasse with alkali and diluted sulfuric acid for bioethanol production. Appl Biochem Biotechnol 155:407–417

Roukas T, Kotzekidou P (1998) Lactic acid production from deproteinized whey by mixed cultures of free and coimmobilized *Lactobacillus casei* and *Lactococcus lactis* cells using fed-batch culture. Enzyme Microb Technol 22:199–204

Runquist D, Hahn-Hägerdal B, Bettiga M (2009) Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. Microb Cell Fact 8:49

Ryckebosch E, Drouillon M, Vervaeren H (2011) Techniques for transformation of biogas to biomethane. Biomass Bioenergy 35:1633–1645

Sathish A, Sims RC (2012) Biodiesel from mixed culture algae via a wet lipid extraction procedure. Bioresour Technol 118:643–647

Schnackenberg J, Ikemoto H, Miyachi S (1996) Photosynthesis and hydrogen evolution under stress conditions in a CO_2-tolerant marine green alga, *Chlorococcum littorale*. J Photochem Photobiol B Biol 34(1):59–62

Schneider H, Wang PY, Chan YK, Maleszka R (1981) Conversion of n-xylose into ethanol by the yeast *Pachysolen tannophilus*. Biotechnol Lett 3:89–92

Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, Smith AG (2010) Biodiesel from algae: challenges and prospects. Curr Opin Biotechnol 21:277–286

Semelsberger TA, Borup RL, Greene HL (2006) Dimethyl ether (DME) as an alternative fuel. J Power Sources 156:497–511

Sheehan J, Cambreco V, Duffield J, Garboski M, Shapouri H (1998) An overview of biodiesel and petroleum diesel life cycles. A report by US Department of Agriculture and Energy, pp 1–35

Shi NQ, Davis B, Sherman F, Cruz J, Jeffries TW (1999) Disruption of the cytochrome c gene in xylose-utilizing yeast *Pichia stipitis* leads to higher ethanol production. Yeast 15:1021–1030

Shi NQ, Cruz J, Sherman F, Jeffries TW (2002) SHAM-sensitive alternative respiration in the xylose-metabolizing yeast *Pichia stipitis*. Yeast 19:1203–1220

Simankova MV, Kotsyurbenko OR, Lueders T, Nozhevnikova AN, Wagner B, Conrad R, Friedrich MW (2003) Isolation and characterization of new strains of methanogens from cold terrestrial habitats. Syst Appl Microbiol 26:312–318

Singh A, Mishra P (1993) Microbial production of ethanol in: microbial pentose utilization: current application in biotechnology. Prog Ind Microbiol 33:147–196

Smith GD, Ewart GD, Tucker W (1992) Hydrogen production by cyanobacteria. Int J Hydrogen Energy 17:695–698

Soderstrom J, Galbe M, Zacchi G (2005) Separate versus simultaneous saccharification and fermentation of two-step steam pretreated softwood for ethanol production. J Wood Chem Technol 25:187–202

Sreenath HK, Koegel RG, Moldes AB, Jeffries TW, Straub RJ (1999) Enzymic saccharification of alfalfa fibre after liquid hot water pretreatment. Process Biochem 35:33–41

Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A et al (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463:559–562

Stelmachowski M, Nowicki L (2003) Fuel from the synthesis gas – the role of process engineering. Applied Energy 74:85–93

Su H, Cheng J, Zhou J, Song W, Cen K (2009) Combination of dark and photo-fermentation to enhance production and energy conversion efficiency. Int J Hydrogen Energy 34:8846–8853

Su H, Cheng J, Zhou J, Song W, Cen K (2010) Hydrogen production from water hyacinth through dark and photo-fermentation. Int J Hydrogen Energy 35:8929–8937

Survase SA, Jurgens G, van Heiningen A, Granström T (2011) Continuous production of isopropanol and butanol using Clostridium beijerinckii DSM 6423. Appl Microbiol Biotechnol 91:1305–1313

Sveshnikov DA, Sveshnikova NV, Rao KK, Hall DO (1997) Hydrogen metabolism of mutant forms of *Anabaena variabilis* in continuous cultures and under nutritional stress. FEMS Microbiol Lett 147:297–301

Swaina PK, Dasa LM, Naik SN (2011) Biomass to liquid: a prospective to challenge research and development in 21st century. Renew Sustain Energy Rev 15:4917–4933

Talebnia F, Pourbafrani M, Lundin M, Taherzadeh MJ (2008) Optimization study of citrus wastes saccharification by dilute acid hydrolysis. BioResour 3:108–122

Tang G, Huang J, Sun Z, Tang Q, Yan C, Liu G (2008) Biohydrogen production from cattle wastewater by enriched anaerobic mixed consortia: influence of fermentation temperature and pH. J Biosci Bioeng 106:80–87

Tavendale MH, Meagher LP, Pacheco D, Walker N, Attwood GT, Sivakumaran S (2005) Methane production from in vitro rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim Feed Sci Technol 123–124:403–419

Vandeska E, Amartey S, Kuzmanova S, Jeffries TW (1996) Fed-batch culture for xylitol production by *Candida boidinii*. Process Biochem 31:265–270

Verho R, Londesborough J, Penttila M, Richard P (2003) Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae*. Appl Environ Microbiol 69:5892–5897

Wahlbom F, Otero RRC, van Zyl WH, Hahn-Hägerdal B, Jonsson LJ (2003) Molecular analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. Appl Environ Microbiol 69:740–746

Wang J, Wan W (2008) Comparison of different pre-treatment methods for enriching hydrogen producing cultures from digested sludge. Int J Hydrogen Energy 33:2934–2941

Wang CC, Chang CW, Chu CP, Lee DJ, Chang VV, Liao CS (2003) Using filtrate of waste biosolids to effectively produce bio-hydrogen by anaerobic fermentation. Water Res 37:2789–2793

Wang D, Xu Y, Zhao G (2004) Fermentation kinetics of different sugars by apple wine yeast *Saccharomyces cerevisiae*. J Inst Brew 110:340–346

Wang YY, Ai P, Hu CX, Zhang YL (2011) Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose. Int Hydrogen Energy 36:390–396

Wellinger A, Lindberg A (1999) Biogas upgrading and utilization. IEA Bioenergy. Task 24: energy from biological conversion of organic waste. http://www.energietech.info/pdfs/Biogas_upgrading.pdf

Wijffels RH, Barbosa MJ (2010) An outlook on microalgal biofuels. Science 329:796–799

Wright SJL, Linton CJ, Edwards RA, Drur E (1991) Isoamyl alcohol (3-methyl-L-butanol), a volatile anti-cyanobacterial and phytotoxic product of some *Bacillus* spp. Lett Appl Microbiol 1991(13):130–132

Xia LM, Sheng XL (2004) High-yield cellulase production by *Trichoderma reesei* in ZU-02 on corncob residues. Bioresour Technol 91:259–262

Xing D, Ren N, Rittmann BE (2008) Genetic diversity of hydrogen-producing bacteria in an acidophilic ethanol-H2-coproducing system, analyzed using the [Fe]-hydrogenase gene. Appl Environ Microbiol 74(4):1232–1239

Xing Y, Li Z, Fan Y, Huo H (2010) Biohydrogen production from dairy manures with acidification pre-treatment by anaerobic fermentation. Environ Sci Pollut Res 17:392–399

Yang S, Tschaplinski TJ, Engle NL, Carroll SL, Martin SL, Davison BH, Palumbo1 AV, Rodriguez M Jr, Brown SD (2009) Transcriptomic and metabolomic profiling of *Zymomonas mobilis* during aerobic and anaerobic fermentations. BioMed Cent Genomics. doi:10.1186/1471-2164-10-34

Yokoi H, Maki R, Hirose J, Hayashi S (2002) Microbial production of hydrogen from starch-manufacturing wastes. Biomass Bioenergy 22:389–395

Yokoyama H, Waki M, Moriya N, Yasuda T, Tanaka Y, Haga K (2007) Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. Appl Microbiol Biotechnol 74:474–483

Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotechnol 20:132–138

Zabut B, El-Kahlout K, Yucel M, Gunduz U, Turker I, Eroglu I (2006) Hydrogen gas production by combined systems of *Rhodobacter sphaeroides* O.U.001 and *Halobacterium salinarum* in a photobioreactor. Int J Hydrogen Energy 31:1553–1562

Zaldivar J, Borges A, Johansson B, Smits HP, Villas-Boas SG, Nielsen J, Olsson L (2002) Fermentation performance and intracellular metabolite patterns in laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 59:436–442

Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio S (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science 267:240–243

Zhao J, Xia L (2010) Ethanol production from corn stover hemicellulosic hydrolysate using immobilized recombinant yeast cells. Biochem Eng J 49:28–32

Zhao QB, Yu HQ (2008) Fermentative H2 production in an up-flow anaerobic sludge blanket reactor at various pH values. Bioresour Technol 99:1353–1358

Zinoviev S, Moler-Langer F, Das P, Bertero N, Fornasiero P, Kaltschmitt M et al (2010) Next-generation biofuels: survey of emerging technologies and sustainability issues. ChemSus Chem 3:1106–1133

Part II

The Interface Between Applied Biocatalysis and Environmental Management

9

Smita Raghava, Seema Rawat,
and Munishwar N. Gupta

Abstract

The early thrust of applied biocatalysis was in the traditional areas of fermentation and food processing. Slowly, as enzymology developed, the applications of enzymes (or whole cells) extended to numerous other areas like textile, detergent, leather and oil and fat industries (Godfrey and West, Industrial enzymology. Macmillan Press Ltd., London, 634 p, 1996; Roy and Gupta, J Biochem Biophys 39:220–228, 2002; Polaina and MacCabe, Industrial enzymes: Structure, functions and applications. Springer Verlag, Dordrecht, 2007). Given their twin virtues of higher rates and specificity, it was natural that biocatalysts started being used in environmental management. The concepts, techniques and some illustrative applications (showing the interface between applied biocatalysis and environment management) form the theme of this chapter. To start with, broad areas wherein applied catalysis has been relevant are discussed.

Keywords

Biocatalysts • Interface • Environment management • Fermentation

Introduction

Bioremediation

This has largely consisted of treating contaminated soil and water using biocatalysts. The discovery of oxygenase for being able to break aromatic ring has been a milestone (Hayashi 1963; Glazer and Nikaido 1995). In the soil, biodegradation of herbicides and pesticides has been an important focus (Parales et al. 2002). It is whole cells which have been mostly used for real applications; enzymology/metabolism in such cases has been studied mostly to understand the molecular level picture of the microbial degradation. Also, this understanding enables one to aim for mutant microorganisms which can do the job more efficiently. In that respect, directed evolution has emerged as a powerful tool. It should be pointed out that microbial oxygenases, in their own right, catalyse reactions which often have considerable synthetic utility, for example, synthesis of trans-diols (Parales et al. 2002). This is a good

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example of close relationship between fundamental and applied aspects profiting from progress in respective areas.

In the case of wastewater, effluents from coal mines and textile industries have attracted early attention. Peroxidases have been widely studied for this application. In real life, because of cost considerations, microbes and microbial consortia have often proved to be the best option (Duran and Esposito 2000).

Biosensors

The interface of a suitable biological catalyst with an appropriate transducer (e.g., an electrode, a chip) constitutes a biosensor (Danielsson and Mosbach 1988). Large number of applications involving control and monitoring of environments have been described in the literature (Rogers and Mascini 1998; Sharpe 2003). The fate of genetically engineered microorganisms in environment, for example, continues to be an important issue (Rogers and Mascini 1998). Two areas, microfluidics (Polson and Hayes 2001) and nanofabrication (Wu and Payne 2004), are reducing the physical size of these devices. That, in turn, is opening up several novel opportunities for their uses in environmental management. Both isolated enzymes and whole cells (Danielsson and Mosbach 1988; Bousse 1996; Simpson et al. 1998) have been used for biosensor applications.

White Biotechnology

This area has evolved out of what has been generally called industrial enzymology (Frazzetto 2003; Glaser 2005; Lorenz and Zink 2005; Ulber and Sell 2007). The thrust here is not to develop 'end-of-the-pipe' technologies for cleanup. Instead, the idea is to replace existing polluting manufacturing practices with greener options. So, rather than waste management, switchover to technology which does not produce waste is the concept. Use of biofuels and biodegradable materials are two well-defined areas of extensive research work (www.mckinsey.com/clientservice/chemicals/pdf/BioVision_Booklet_final.pdf; Jenck et al. 2004). The concept of biorefinery (wherein, all building blocks for synthesis of chemicals/drugs/materials would be derived from renewable sources) is one of the most powerful ideas (Gupta and Raghava 2007; http://www.epobio.net/pdfs/0611CellWallSaccharificationReport_c.pdf) to emerge in recent times and would impact the environmental sciences considerably. The use of biocatalysts instead of chemical catalysts is an important plank of white biotechnology. What is perhaps less well known is the importance of the use of microwaves (Roy and Gupta 2003a) instead of conventional heating in this context. The US technology vision document emphasises this importance (http://www.ccrhq.org/vision/index.html).

There is an important aspect of white biotechnology which deserves greater attention than it gets in the context of environment management. There are cases where a particular product is required and produced in large amounts. Often, there is a by-product which is coproduced. It could be considered a waste or may not have sufficient applications. In such cases, biotechnology has sometimes turned this vexing problem into a tremendous opportunity. Before we illustrate this idea with two important examples, some of the important concepts and developments which are impacting environmental managements are outlined. There is a need for environmental biotechnologists to be more familiar with these so that these can be applied more extensively.

Sourcing Biocatalysts

Both enzymes and whole cells have been used in environmental monitoring and control. Working with enzymes is comparatively a costlier business since downstream processing costs constitute a major share (60–90%) of the overall production costs. However, the specific activity and hence overall rates/conversion factors are quite high. Using whole cells directly saves the cost of separating the enzyme (activity). There

may be an interference with other enzymes present in the cell. The rates obtained may be low especially if the mass transfer constraints dominate (this would be especially significant during biodegradation of biomass which is mostly macromolecular in nature). Whole cell biocatalysis is less predictable. Nevertheless, it has been the method of choice in bioremediation (Dua et al. 2002). In the context of both types of biocatalysts, large diversity is available. Metagenomics makes it possible to source enzymes from the organisms which cannot even be cultivated (Handelsman 2004; Yun and Ryu 2005; Langer et al. 2006; Ferrer et al. 2007). Protein engineering (Palackal et al. 2004), gene shuffling (Stemmer 1994a, b) and directed evolution (Arnold et al. 2003; Otten and Quax 2004; Williams et al. 2004) allow one to modify a given biocatalyst to endow it with any desired property. Thermal stability, stability towards organic solvents, substrate specificity, regioselectivity and enantioselectivity all can be engineered via molecular biology techniques. Metabolic engineering now has come of age and cell factories have emerged as a powerful concept (Koffas and Cardayre 2005; Yun and Ryu 2005).

As far as sourcing enzymes from natural sources is concerned, microbial sources dominate the scene (even in the case of recombinant sources, microbes constitute most often used host systems for expression of recombinant DNA). There is an increasing emphasis on sourcing enzymes from extremophiles (Madigan 2000; Rossi et al. 2003; Podar and Reysenbach 2006). Considering that their *milieu* for environmental applications is likely to be harsh, extremophiles constitute an ideal source for isolating the required biocatalysts. High temperature, high salinity or extreme pH conditions are some of the conditions which environmental applications are likely to encounter.

The separation and purification of enzymes is an area which has seen tremendous developments in recent years (Table 9.1). Traditional multistep protocols are being increasingly replaced by shorter and mostly affinity-based protocols. Today, the most often used purification approach uses affinity tags (like polyhistidine (Gaberc-Porekar and Menart 2001), cellulose-binding domain (Tomme et al. 1998) or maltose-binding protein (Riggs 1990)) to purify recombinant proteins. The interfacing of the affinity concept with precipitation (Roy and Gupta 2003b; Mondal et al. 2006), aqueous two-phase extraction (Teotia et al. 2004) and expanded bed chromatography (Roy et al. 1999) has given some very powerful and efficient technologies. A new technique macro-(affinity ligand) facilitated three-phase partitioning (MLFTPP) has been recently described and has already found quite a few applications (Table 9.2). While chromatographic methods would continue to be used for high purity preparations (such as pharmaceutical proteins), most of the enzymes required for biosensors, bioremediation and bioconversions do not require high purity and can be easily and economically obtained by non-chromatographic methods. Currently, membrane-based separations are already used at industry level, but other non-chromatographical methods also would ultimately attain industrial maturity (Przybycien et al. 2004). This would make enzyme-based technologies considerably cheaper.

Table 9.1 Summary of chromatographic and non-chromatographic techniques used in protein purification (Roy et al. 2007)

Technique	Examples
Chromatographic	Ion-exchange chromatography
	Hydrophobic interaction chromatography
	Affinity chromatography
	Gel-filtration chromatography
	Radial flow chromatography
	Perfusion chromatography
	Expanded bed chromatography
	Displacement chromatography
	Monoliths
Non-chromatographic	Aqueous two-phase extraction
	Three-phase partitioning
	Reverse micellar extraction
	Crossflow ultrafiltration
	Preparative electrophoresis

Table 9.2 Enzymes purified by macro-(affinity ligand) facilitated three-phase partitioning (MLFTPP)

Enzyme	Macro-(affinity ligand)	Activity recovered (%)	Fold purification	Reference
Xylanase	Eudragit S-100	60	95	Sharma and Gupta (2002)
α-amylase from porcine pancreas	Esterified alginate	92	10	Mondal et al. (2003a)
α-amylase from *B. amyloliquefaciens*	Esterified alginate	74	5.5	Mondal et al. (2003a)
Wheat germ amylase	Esterified alginate	77	55	Mondal et al. (2003a)
Glucoamylase	Esterified alginate/ alginate	83	20	Mondal et al. (2003b)
Pullulanase	Esterified alginate	89	38	Sharma et al. (2003)
Pectinase	Alginate	96	13	Sharma et al. (2003)
Cellulase	Chitosan	92	16	Sharma et al. (2003)

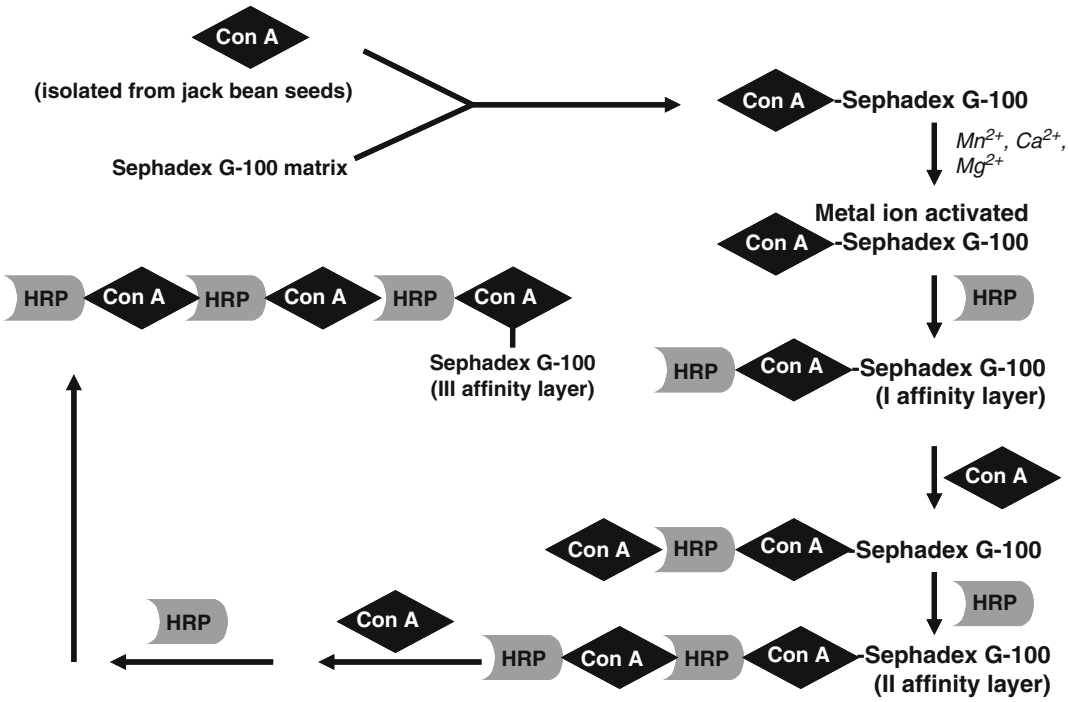


Fig. 9.1 Preparation of affinity-layered horseradish peroxidase (HRP)

Immobilisation and Reusability

Immobilisation of biocatalysts is carried out to obtain reusable biocatalysts. While large number of approaches (such as noncovalent methods, covalent methods, entrapment and encapsulation, bioaffinity immobilisation) have been described in the literature (Cao 2006; Guisan 2006), few relatively less exploited and recent approaches are worth mentioning.

The technique of affinity layering exploits bioaffinity immobilisation (Mattiasson 1988; Saleemuddin 1999) and allows one to deposit large amount of biocatalytic activity over a small surface (Fig. 9.1). Its application to both biosensors and biocatalysis area has been described (Farooqui et al. 1997a, b; Dalal and Gupta 2007). Nanosized matrices especially nanotubes constitute attractive matrices and offer some special advantages in biosensor applications (Davis et al.

1998; Sayler et al. 2004). Hence, immobilisation on such surfaces has attracted considerable attention in recent years (Jiang et al. 2004; Shah and Gupta 2007a; Shah et al. 2007). Some recent designs such as CLEC (cross-linked enzyme crystals (St. Clair and Navia 1992)) and CLEA (cross-linked enzyme aggregates (Wilson et al. 2004; Sheldon et al. 2005; Shah et al. 2006)) do not use any matrices and hence lead to high space-time yields for bioconversions, biotransesterification and bioremediation. Combi-CLEAs (Dalal et al. 2006) and multipurpose CLEAs (Dalal et al. 2007) constitute some attractive emerging concepts in this regard.

Nonaqueous Enzymology

It is now well established that enzymes can be used in nearly anhydrous organic media (Gupta 1992, 2000; Gupta and Roy 2004), ionic liquids (Sheldon 2001; Sheldon et al. 2002; van Rantwijk et al. 2003; Shah and Gupta 2007b, c), reverse micelles (Luisi 1985; Luisi et al. 1988; Martinek 1989) and even aqueous-organic cosolvent mixtures (Antonini et al. 1981). This opens up several possibilities. To start with, many hydrophobic substrates can be dissolved in such media. Many hydrolases can be used for synthetic purposes. Most relevant to present discussion is the use of biosensors like thermistors in nonaqueous *milieu* (Ramanathan et al. 2000; Ramanathan and Danielsson 2001).

Bioconversion/Biotransformation of a 'Waste' into a Value-Added Product

Whey Hydrolysis

Whey is a by-product in cheesemaking. It contains small amounts of high-quality proteins; otherwise, it is essentially a lactose solution (~4.7%, w/v). The enzyme lactase (β-D-galactoside galactohydrolase; E.C.3.2.1.23) occurs widely and has been known for a long time as an enzyme which hydrolyses lactose to glucose and galactose (Huber et al. 1994). As early as 1950, isolation of lactase activity and microbial sources led to the proposal that the enzyme may be useful in dairy industry. Stimpson in 1957 patented application of lactose-hydrolysed whey in animal feed. Around 1960s, lactose intolerance in humans was discovered (Dahlqvist and Lindquist 1971; Kretchmer 1972). This led to intensified interest in lactose for producing low-lactose milk. This coincided with significant rise in the price of sugar in Europe. This again led to resurgence in the interest in hydrolyzing whey lactose to the constituent sugars (Wigley 1996). By 1976, 36.4 billion pounds of whey was being produced in the USA (Holsinger 1978). In early years, disposal of whey constituted a huge problem. When discharged into water, it has a high biological oxygen demand (BOD) value and was considered a serious pollutant. Over the years, whey lactose and whey proteins both have emerged as rich sources of variety of industrial products. The proteins are hydrolysed by proteases and are used in sports drinks, etc. (Liese and Filho 1999). The disaccharide is hydrolysed by lactases. The two most commonly used lactases at commercial levels are from *Aspergillus oryzae* and *Kluyveromyces lactis*. The importance of hydrolysis of constituent monosaccharides arises from the several disadvantages of using lactose as such as a sweetener. It is less sweet than the monosaccharides, has sandy 'mouthfeel' if used as a sweetener, has poor solubility (about 20 g/100 g water at room temperature) and has poor fermentability. The last feature means that not many microorganisms can utilise this as an energy source.

One of the earliest pilot plants based upon lactase technology for treatment of whey was established at Connecticut/Lehigh Universities in the USA. It used an *A. niger* lactase adsorbed to porous alumina carrier. The fluidised bed reactor used whey ultrafiltered permute as the feed. Industrial Keymen Laakso Dairy (Finland) used *A. niger* lactase adsorbed on a phenol formaldehyde resin Duolite ES-762. A pilot plant in Germany (West) used covalently immobilised *A. oryzae* enzyme for processing of acid whey. Similarly, Sumitomo (Japan) and Amerace Corp. (USA) pilot plants used covalently immobilised *A. oryzae* lactase for processing of whey (Gekas and Lopez-Leiva 1985; Kosaric and Asher 1985).

Two major applications of hydrolysed whey are as an industrial sweetener and for further

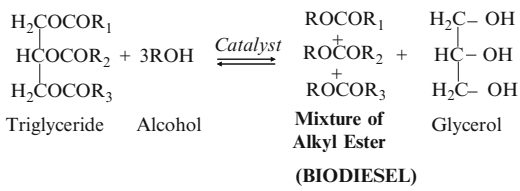
fermentation to alcohol and other valuable chemicals. While in the UK, whey syrup is mostly used to replace sweet condensed milk, in Finland, it finds large applications in bakeries and into a flavoured whey drink! Various other applications reported are in confectionary products, desserts and ice creams, spreads, dressings, soft drinks and pet foods (Wigley 1996).

While the above technology revolves around this hydrolytic activity of lactase, it should be added that lactose, using the same β-galactosidase (lactase), can also be used to synthesise oligosaccharides. These galactooligosaccharides are used as probiotic food ingredients (Modler et al. 1990). A probiotic substance is a nondigestible food ingredient that selectively stimulates the growth of beneficial bacterial species already present in the colon (Modler et al. 1990). The establishment of this *bifidus* microflora is claimed to have tremendous health benefits. Such substances have been legislated in Japan in 1991 as FOSHU (Foods for Specified Health Use) (Shortt 1999). These are now beginning to attract considerable attention in other parts of the world. Major companies involved in galactooligosaccharide production are Yakult Honsha, Nissin Sugar Products Manufacturing Company and Snow Brand Milk Products (all in Tokyo, Japan).

Hence, the utilisation of whey lactose constitutes one of the early and successful examples of biotechnology converting an environmental problem into a series of processes which can yield large number of value-added products and commodities.

Conversion of Glycerol

A large amount of glycerol is generated as a by-product of biodiesel. Biodiesel consists of mono-alkyl esters of fatty acids and is produced by transesterification of fats/oils with methanol or ethanol.



Biodiesel is one of the biofuels and this technology has been developed to substitute petroleum products. Conventional diesel engines can run on biodiesel without any major modification of the engine. This is also an environment-friendly fuel. Hence, in the USA alone, the production of biodiesel has increased from approximately 75 million gallons in 2005 to >250 million gallons in 2006 and is expected to cross 300 million gallons in 2007. The annual total production capacity in USA is projected to be approximately 2 billion gallons by 2009 (Johnson and Taconi 2007). The starting oil could be any vegetable oil like soybean oil. In countries like India, wherein edible oils are in short supply, nonedible oil like from *Jatropha curcas* has been identified as the major starting material (Francis et al. 2005). All this global emphasis on biodiesel production has led to the glycerol glut. For every 3 moles of biodiesel, one mole of glycerol is produced. It is reported that the global glycerol market was 800,000 t in 2005, with 400,000 t from biodiesel alone (Pagliaro et al. 2007). The situation is so serious that many plants which were producing glycerol (from propylene via epichlorohydrin) are closing. Again both chemists and biotechnologists are trying to convert this 'threat' to an 'opportunity'. A large variety of chemicals and biotechnological routes are being developed to convert glycerol into value-added products. Some value-added products which can be obtained from glycerol by biocatalytic/fermentative approach are listed below:

- Dihydroxyacetone: A main ingredient of sunscreen skin care products has a market of 2,000 t/year. It is currently produced by microbial fermentation by *Gluconobacter oxydans* (Pagliaro et al. 2007).
- 1,3-Propanediol: The new generation textile films, Sonora™ and Corerra™, are produced from 1,3-propanediol (and terephthalic acid). Bacterial strains of *Citrobacter*, *Enterobacter*, *Ilyobacter*, *Klebsiella*, *Lactobacillus*, *Pelobacter* and *Clostridium* are able to convert glycerol into 1,3-propanediol (Papanikolaou et al. 2002; Lin et al. 2005).
- Butanol: This alcohol has a higher energy yield per gallon as compared to ethanol.

Clostridium pasteurianum is reported to convert crude glycerin to butanol by anaerobic fermentation (Biebl et al. 1992; Biebl 2001).

- Ethanol: A start-up company called Glycos Biotechnologies is already in place and uses *Escherichia coli* to convert glycerin to ethanol, again by anaerobic fermentation (Coombs 2007).

While above products are already at commercial level production stage, several other potential products are also possible. Species of *Yarrowia*, *Candida* and *Rhodotorula* are capable of converting glycerol into citric acid (Papanikolaou and Aggelis 2002; Johnson and Taconi 2007), sophorolipids (Ashby et al. 2005) and single cell oil (Johnson and Taconi 2007). The acidogenesis + methanogenesis of glycerol can produce biogas. *Enterobacter aerogenes* is also reported to convert glycerol into H_2 and ethanol (Johnson and Taconi 2007). Anaerobic fermentation of glycerol by *Clostridia* has been attracting considerable attention. Variety of products, acetic acid, butyric acid, lactic acid, succinic acid, butanol, ethanol and acetone, are produced at various concentrations. Some of these are key platform chemicals (Johnson and Taconi 2007).

It has been pointed out that price of crude glycerin (of the quality obtained during biodiesel production) is 600 €/t. Glycerol is fast emerging as an important raw material for chemical industries.

The utilisation of crude glycerol for producing industrial chemicals is likely to be an important component of the biorefinery concept. This concept seeks to replace existing petroleum-based industries by industries which would be ultimately run by using chemicals desired from renewable plant resources (Clark et al. 2006).

Conclusion

The interface between biology and environmental sciences is rather extensive and a rich one. In this chapter, an effort has been made to focus on developments/concepts in applied biocatalysis which workers in traditional areas of environmental science tend to overlook. To go beyond where we are would require cross-fertilisation of ideas. Hopefully, this chapter would encourage some useful steps in that direction.

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References

Antonini E, Carrea G, Cremonesi P (1981) Enzyme catalysed reactions in water-organic solvent two-phase systems. Enzyme Microb Technol 3:291–296

Arnold FH, Georgiou G, Totawa NJ (2003) Directed enzyme evolution: screening and selection methods. In: Arnold FH, Georgiou G (eds) Methods in molecular biology. Humana Press, Totowa

Ashby RD, Nunez A, Solaiman DKY, Fogila TA (2005) Sophorolipid biosynthesis from a biodiesel co-product stream. J Am Oil Chem Soc 82:625–630

Biebl H (2001) Fermentation of glycerol by *Clostridium pasteurianum* – batch and continuous culture studies. J Ind Microbiol Biotechnol 27:18–26

Biebl H, Marten S, Hippe H, Deckwer WD (1992) Glycerol conversion to 1, 3-propanediol by new strains of *Clostridium butyricum*. J Ind Microbiol Biotechnol 15:498–502

Bousse L (1996) Whole cell biosensors. Sensors Actuators 34:270–275

Cao L (2006) Carrier bound immobilized enzymes: principles, applications and design. Wiley-Vch Verlag Gmbh and Company, Weinheim, 563 p

Clark JH, Budarin V, Deswarte FEI, Hardy JJE, Kerton FM, Hunt AJ, Luque R, Macquarrie DJ, Milkowski K, Rodriguez A, Samuel O, Taveneer SJ, White RJ, Wilson AJ (2006) Green chemistry and the biorefinery – a partnership for a sustainable future. Green Chem 10:853–860

Coombs A (2007) Glycerin bioprocessing goes green. Nat Biotechnol 25:953–954

Dahlqvist A, Lindquist B (1971) Lactose intolerance and protein malnutrition. Acta Paediatr Scand 60:488–494

Dalal S, Gupta MN (2007) Treatment of phenolic wastewater by horseradish peroxidase immobilized by bioaffinity layering. Chemosphere 67:741–747

Dalal S, Kapoor M, Gupta MN (2006) Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions. J Mol Catal B: Enzym 44:128–132

Dalal S, Sharma A, Gupta MN (2007) A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulose activities. Chem Cent J 1:16

Danielsson B, Mosbach K (1988) Enzyme thermistors. In: Mosbach K (ed) Methods in enzymology, vol 137. Academic, Orlando, pp 181–197

Davis JJ, Green MLH, Hill HAO, Leung YC, Sadler PJ, Sloan J, Xavier AV, Tsang SC (1998) The immobilization of proteins in carbon nanotubes. Inorg Chem Acta 272:261–266

Dua M, Singh A, Sethunathan N, Johri A (2002) Biotechnology and bioremediation: successes and limitations. Appl Microbiol Biotechnol 59:143–152

Duran N, Esposito E (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment. Appl Catal B Environ 28:83–99

Farooqui M, Saleemuddin M, Ulber R, Sosnitza P (1997a) Bioaffinity layering: a novel strategy for the immobilization of large quantities of glycoenzymes. J Biotechnol 55:171–179

Farooqui M, Sosnitza P, Saleemuddin M, Ulber R (1997b) Immunoaffinity layering of enzymes. Appl Microbiol Biotechnol 52:373–379

Ferrer M, Beloqui A, Golyshin PN (2007) Microbial metagenomes: moving forward industrial biotechnology. J Chem Technol Biotechnol 82:421–423

Francis G, Edinger R, Becker K (2005) A concept for simultaneous wasteland reclamation, fuel production and socio-economic development in degraded areas in India: Need, potential, and perspective of *Jatropha* plantation. Nat Res Forum 29:12–24

Frazzetto G (2003) White biotechnology. EMBO Rep 4:835–837

Gaberc-Porekar V, Menart V (2001) Perspectives of immobilized metal affinity chromatography. J Biochem Biophys Methods 49:335–360

Gekas V, Lopez-Leiva M (1985) Hydrolysis of lactose: a literature review. Process Biochem 20:2–12

Glaser JA (2005) White biotechnology. Clean Technol Environ Policy 7:233–235

Glazer AN, Nikaido H (1995) Environmental applications. In: Glazer AN, Nikaido H (eds) Microbial biotechnology: fundamentals of applied microbiology. W.H. Freeman and Company, New York, pp 561–614

Godfrey T, West S (1996) Industrial enzymology. Macmillan Press Ltd., London, 634 p

Guisan JM (2006) Immobilization of enzymes and cells. In: Guisan JM (ed) Methods in biotechnology. Humana Press, Totowa, 449 p

Gupta MN (1992) Enzyme function in organic solvents. Eur J Biochem 203:25–32

Gupta MN (ed) (2000) Methods in non-aqueous enzymology. Birkhauser, Basel

Gupta MN (ed) (2002) Methods for affinity based separation of enzymes and proteins. Birkhäuser, Basel, 225 p

Gupta MN, Raghava S (2007) Relevance of chemistry to white biotechnology. Chem Cent J 1:17

Gupta MN, Roy I (2004) Enzymes in organic media: forms, function and applications. Eur J Biochem 271:1–9

Handelsman J (2004) Metagenomics: applications of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 6:669–685

Hayashi O (1963) Direct oxygenation by O2, oxygenases. In: Boyer P, Lardy H, Myrbäck K (eds) The enzymes, vol 8. Academic, New York, p 353

Holsinger VH (1978) Application of lactose-modified milk and whey. Food Technol 32:35–40

Huber RE, Gupta MN, Khare SK (1994) The active site and mechanism of β- galactosidase from *Escherichia coli*. Int J Biochem 26:309–318

Jenck JF, Agterberg F, Droescher MJ (2004) Products and processes for a sustainable chemical industry: a review of achievements and prospects. Green Chem 6:544–556

Jiang K, Schadler LS, Segel RW, Zhang X, Zhang HA, Terrones M (2004) Protein immobilization on carbon nanotubes via a 2-step process of diimide-activated amidation. J Mater Chem 14:37–39

Johnson DT, Taconi KA (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog 26:338–348

Koffas M, Cardayre SD (2005) Evolutionary metabolic engineering. Metab Eng 7:1–3

Kosaric N, Asher YJ (1985) The utilization of cheese whey and its components. In: Fiether A (ed) Advance in biochemical engineering, vol 19. Springer, New York, pp 25–60

Kretchmer N (1972) Lactose and lactase. Sci Am 227:271–78

Langer M, Gabor EM, Liebeton K, Meurer G, Niehaus F, Schulze R, Eck J, Lorenz P (2006) Metagenomics: an inexhaustible access to nature's diversity. Biotechnol J 1:815–821

Liese A, Filho MV (1999) Production of fine chemicals using biocatalysis. Curr Opin Biotechnol 10:595–603

Lin R, Liu H, Hao J, Cheng K, Liu D (2005) Enhancement of 1, 3-propanediol Production by *Klebsiella pneumoniae* with fumarate addition. Biotechnol Lett 27:1755–1759

Lorenz P, Zink H (2005) White biotechnology: differences in US and EU approaches? Trends Biotechnol 23:570–574

Luisi PL (1985) Enzymes hosted in reverse micelles in hydrocarbon solution. Angew Chem Int Ed 24:439–450

Luisi PL, Giomini M, Pileni MP, Robinson BH (1988) Reverse micelles as hosts for proteins and small molecules. Biochem Biophys Acta 974:209–246

Madigan MT (2000) Extremophilic bacteria and microbial diversity. Ann Mo Bot Gard 87:3–12

Martinek K (1989) Micellar enzymology: potentialities in fundamental and applied areas. Biochem Int 18:871–893

Mattiasson B (1988) Affinity immobilization. In: Mosbach K (ed) Methods in enzymology, vol 137. Academic, New York, pp 647–656

Modler HW, Mckellar RC, Yaguchi M (1990) Bifidobacteria and bifidogenic factors. Can Inst Food Sci Technol 23:29–41

Mondal K, Sharma A, Gupta MN (2003a) Macro-(affinity ligand) facilitated three phase partitioning (MLFTPP) of α-amylases using modified alginate. Biotechnol Prog 19:493–494

Mondal K, Sharma A, Gupta MN (2003b) Macro-(affinity ligand) facilitated three phase partitioning (MLFTPP) for purification of glucoamylase and pullulanase using alginate. Protein Expr Purif 28:190–195
Mondal K, Roy I, Gupta MN (2006) Affinity based strategies for protein purification. Anal Chem 78:3499–3504
Otten LG, Quax WJ (2004) Directed evolution: selecting today's biocatalysis. Biomol Eng 22:1–9
Pagliaro M, Ciriminna R, Kimura H, Rossi M, Pina CD (2007) From glycerol to value-added products. Angew Chem Int Ed 46:4434–4440
Palackal N, Brennan Y, Callen WN, Dupree P, Frey G, Goubet F, Hazlewood GP, Healey S, Kang YE, Kretz KA, Lee E, Tan X, Tomlinson GL, Verruto J, Wong VW, Mathur EJ, Short JM, Robertson DE, Steer BA (2004) An evolutionary route to xylanase process fitness. Protein Sci 13(2):494–503
Papanikolaou S, Aggelis G (2002) Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single stage continuous culture. Bioresour Technol 82:43–49
Papanikolaou P, Muniglia L, Chevalot G, Aggelis G, Marc I (2002) *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. J Appl Microbiol 92:737–744
Parales RE, Bruce NC, Schmid A, Wackett LP (2002) Biodegradation, biotransformation and biocatalysis. Appl Environ Microbiol 68:4699–4709
Podar M, Reysenbach AL (2006) New opportunities revealed by biotechnological explorations of extremophiles. Curr Opin Biotechnol 17:250–255
Polaina J, MacCabe AP (eds) (2007) Industrial enzymes: structure, functions and applications. Springer, Dordrecht, 639 p
Polson NA, Hayes MA (2001) Controlling fluids in small places: microfluidics. Anal Chem 73:312A–319A
Przybycien TM, Pujar NS, Steele LM (2004) Alternative bioseparation operations: life beyond packed-bed chromatography. Curr Opin Biotechnol 15:469–478
Ramanathan K, Danielsson B (2001) Principles and applications of thermal biosensors. Biosensors and bioelectronics. Protein Sci 16:417–423
Ramanathan K, Jönsson BR, Danielsson B (2000) Analysis in non-aqueous milieu using thermistors. In: Gupta MN (ed) Methods in non-aqueous enzymology. Birkhauser, Basel, pp 174–194
Riggs PD (1990) In: Ausebel FM, Brent R, Kingston RE, Moorey DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York. pp 16.6.1–16.6.10
Rogers KR, Mascini M (1998) Biosensors for field analytical monitoring. Field Anal Chem Technol 2:317–331
Rossi M, Ciaramella M, Cannio R, Pisani FM, Moracci M, Bartolucci S (2003) Extremophiles 2002. J Bacteriol 185:3683–3690
Roy I, Gupta MN (2002) Applied biocatalysis: an overview. Indian J Biochem Biophys 39:220–228.
Roy I, Gupta MN (2003a) Applications of microwaves in biological sciences. Curr Sci 85:1685–1693
Roy I, Gupta MN (2003b) Smart polymeric materials: emerging biochemical applications. Chem Biol 10: 1161–1171
Roy I, Pai A, Lali A, Gupta MN (1999) Comparison of batch, packed bed and expanded bed purification of *A. niger* cellulase using cellulose beads. Bioseparation 8:317–326
Roy I, Mondal K, Gupta MN (2007) Leveraging protein purification strategies in proteomics. J Chromatogr B 849:32–42
Saleemuddin M (1999) Bioaffinity based immobilization of enzymes. Adv Biochem Eng Biotechnol 64:203–226
Sayler GS, Simpson ML, Cox CD (2004) Emerging foundations: nano-engineering and bio-microelectronics for environmental biotechnology. Curr Opin Microbiol 7:267–273
Shah S, Gupta MN (2007a) Simultaneous refolding, purification and immobilization of xylanase with multiwalled carbon nanotubes. Biochem Biophys Acta 1784(2):363–367. doi:10.1016/j.bbapap. 2007.11.015
Shah S, Gupta MN (2007b) Obtaining high transesterification activity for subtilisin in ionic liquids. Biochem Biophys Acta (Gen Subj) 1770:94–98
Shah S, Gupta MN (2007c) Kinetic resolution of (±)-1-phenylethanol in [Bmim][PF6] using high activity preparations of lipases. Bioorg Med Chem Lett 17:921–924
Shah S, Sharma A, Gupta MN (2006) Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. Anal Biochem 351:207–213
Shah S, Solanki K, Gupta MN (2007) Enhancement of lipase activity in non-aqueous media upon immobilization on multi-walled carbon nanotubes. Chem Cent J 1:30
Sharma A, Gupta MN (2002) Macro-(affinity ligand) facilitated three phase partitioning (MLFTPP) for purification of xylanase. Biotechnol Bioeng 80:228–232
Sharma A, Mondal K, Gupta MN (2003) Separation of enzymes by sequential macroaffinity ligand-facilitated three-phase partitioning. J Chromatogr A 995:127–134
Sharpe M (2003) It's a bug's life: biosensors for environmental monitoring. J Environ Monit 5:109–113
Sheldon RA (2001) Catalytic reactions in ionic liquids. Chem Commun 23:2399–2407
Sheldon RA, Lau RM, Sorgedrager MJ, van Rantwijk F, Seddon KR (2002) Biocatalysis in ionic liquids. Green Chem 4:147–151
Sheldon RA, Sorgedrager MJ, van Rantwijk F (2005) Cross-linked enzyme aggregates: a novel and versatile method for enzyme immobilization. Biocatal Biotransformation 23:141–147
Shortt C (1999) The probiotic century: historical and current perspectives. Trends Food Sci Technol 10:411–417
Simpson ML, Sayler GS, Applegate BM, Ripp S, Nivens DE, Paulus MJ, Jellison GE (1998) Bioluminescent-bioreporter integrated circuits form novel whole-cell biosensors. Trends Biotechnol 16:332–338
St. Clair NL, Navia MA (1992) Cross-linked enzyme crystals as robust biocatalysts. J Am Chem Soc 114:7314–7316
Stemmer WP (1994a) Rapid evolution of a protein in vitro by DNA shuffling. Nature 370:389–391
Stemmer WP (1994b) DNA shuffling by random fragmentation and reassembly: in vitro recombination

for molecular evolution. Proc Natl Acad Sci U S A 91:10747–10751

Stimpson E (1957) Lactase-hydrolyzed lactose in feed. US Patent No. 2.781.266

Teotia S, Lata R, Gupta MN (2004) Chitosan as a macro-(affinity ligand): Purification of chitinases by affinity precipitation and aqueous two-phase extraction. J Chromatogr A 1052:85–91

Tomme P, Boraston A, McLean B, Kormos J, Creagh AL, Sturch K, Gilkes NR, Haynes CA, Warren RA, Kilburn DG (1998) Characterization and affinity applications of cellulose-binding domains. J Chromatogr B 715:283–296

Ulber R, Sell D (eds) (2007) White biotechnology. Springer, Berlin

van Rantwijk F, Lau RM, Sheldon RA (2003) Biocatalytic transformations in ionic liquids. Trends Biotechnol 21:131–138

Wigley RC (1996) Cheese and whey. In: Godfrey T, West S (eds) Industrial microbiology. Macmillan Press Ltd, London, pp 134–154

Williams GJ, Neloson AS, Berry A (2004) Directed evolution of enzymes for biocatalysis and the life sciences. Cell Mol Life Sci 61:3034–3046

Wilson L, Betancor L, Ferandez-Lafuente G, Fuentes M, Hidalgo A, Guisan JM, Pessela C, Ferandez-Lafuente R (2004) Cross-linked aggregates of multimeric enzymes: a simple and effective methodology to stabilize their quaternary structure. Biomacromolecules 5:814–817

Wu LQ, Payne GF (2004) Biofabrication: using biological materials and biocatalysts to construct nanostructured assemblies. Trends Biotechnol 22:593–599

Yun J, Ryu S (2005) Screening for novel enzymes from metagenome and SIGEX, as a way to improve it. Microb Cell Fact 4:8

Metagenomics: Mining Environmental Genomes

10

Sheela Srivastava, Nitika Ghosh, and Gargi Pal

Abstract

The use of traditional microbiological culturing methods for the study of microbes had limited success since it has been estimated that 99% of microbes cannot be cultivated easily. Over the past decade, "metagenomics," which is the culture-independent genomic analysis of microbes, has been developed to overcome these difficulties. Metagenomic analysis involves the basic steps like (1) the selection of an environmental niche, (2) the isolation of genetic material directly from an environmental sample, (3) manipulation of the genetic material, (4) library construction, and (5) the analysis of genetic material in the metagenomic library. The screening of clones can be done for phylogenetic markers or for other conserved genes by hybridization or multiplex PCR or for expression of specific traits, such as enzyme activity or antibiotic production, or they can be sequenced randomly. This chapter gives an overview of metagenomics including its success as well as future biotechnological applications in pharmaceuticals, bioactive molecules, biocatalysts, biomaterials, and others. There can be little doubt that the field of metagenomics gene discovery offers enormous scope and potential for both fundamental microbiology and biotechnological development.

Keywords

Metagenomics • Library construction • Screening • Sequence-based and function-based analysis

Introduction

Microbes, being ubiquitous in nature, are essential for every part of human life. It is through the capacity of these tiny minuscules that our planet is replenished with the key elements of life, such as carbon, nitrogen, oxygen, phosphorous, and sulfur in accessible forms. All plants and animals have closely associated microbial communities that provide them necessary nutrients, growth factors, and vitamins. The billions of benign microbes that live in the human gut help us to digest food, break down toxins, and fight off disease-

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causing counterparts. We also depend on microbes to clean up pollutants in the environment, such as oil and chemical spills. Microbial communities not only play key role in maintaining environmental quality and health but also participate in the upkeep of individual plants and animals. They can also live in extreme environments, at temperatures, pressure, and pH levels in which no other life forms can dare to tread.

Think about the countless jobs these tiny things do for us, starting from making antibiotics, many drugs in clinical use, enzymes, and other chemicals for industrial use to remediate pollutants in soil and water, to enhance crop productivity, to produce biofuels, to ferment Variety of foods, and to provide unique signatures that form the basis of microbial detection in disease diagnosis and forensic analysis. This is an endless list, and knowing them well not only includes all the processes involved and the genes responsible for all kinds of work they do but also tapping the vast genetic diversity existing in the microbial world. It is, therefore, no surprise that more than 900 genomes have already been sequenced.

We currently have little information (<1% of all bacterial species) on the vast majority of microorganisms present in Earth's different environments, mainly due to our inability to culture them in the laboratory. Historically, our inability to culture microorganisms is due to lack of knowledge of their physiology and environmental cues that may help in designing suitable culture medium. However, new cultivation techniques are beginning to address this problem (Handelsman 2004). Nonetheless, most of our knowledge has been gleaned from the relatively small number of presently culturable representatives. So miniscule is this representation that it gives no clues as to what constitutes a microbial world and what all can it do.

Going back to nature, therefore, what do we really want to know in microbial ecology and evolution? The fundamental questions include what types of species are present (phylogenetic questions), how many of each type are present at a given time and location (genomic questions), what are they doing there (metabolic and functional questions), and what resources are they using (biogeochemical questions)? The realization that most microorganisms cannot be grown readily in pure culture forced microbiologists to question their belief that the microbial world had been conquered.

Microbiology has experienced a number of transformations during its history of three and a half century. Each of these stages has altered microbiologists' view of microorganisms and how to study them (Handelsman 2004). The roots of microbiology are firmly associated with the invention of microscope. The first record of a human being seeing a bacterial cell dates back to 1663, when Antonie van Leeuwenhoek watched bacteria that he recovered from his own teeth through his homemade microscope. He was a keen observer and an outstanding combiner of ground glass pieces to obtain a magnified view of a sample. His observations and detailed illustrations of microbial life prompted many other observers (both scientists and nonscientists) to take an interest in the microscopic world. His colorful descriptions of bacteria made their study compelling; in his descriptions of the many shapes of the bacteria he sampled from his teeth, he marveled that one "shot through the water like a pike does through water," firmly establishing that these tiny objects were indeed alive. For the next 200 years, refinement in microscopy enabled microbiologists to view heterotrophs, autotrophs, and obligate parasites alike with better details. Robert Koch's postulates and his own innovation in developing culture media were instrumental in making another shift. From 1880s forward, the microbiological world was virtually restricted to the culture tubes of a microbiology laboratory.

Microbiologists were attracted to the power and precision of studies of bacteria in pure culture, and as a result, most of the knowledge that fills modern microbiology textbooks today is derived from organisms maintained in pure culture. Because culturing provided the platform for building the depth and details of modern microbiological knowledge, for a long time microbiologists ignored the challenge to identify and characterize uncultured organisms. They focused instead on the rich source of diversity found in the readily culturable model organisms, and this contributed to the explosion of knowledge in microbial physiology and genetics in the 1960s to mid-1980s. Meanwhile, the study of uncultured

microorganisms remained in the hands of a few persistent enthusiasts who began to accumulate hints that flitted at the edge of the microbiological consciousness, suggesting that culturing did not capture the full spectrum of microbial diversity. Many of the organisms could not be cultured on agar medium because their temperature requirements exceeded the melting point of the agar. Or that we did not, and still do not, understand their growth requirements. Therefore, elucidating the physiological function of microorganisms without culturing them required ingenuity. One of the indicators that cultured microorganisms did not represent much of the microbial world was the often observed "great plate count anomaly" – the discrepancy between the sizes of populations estimated by dilution plating and by microscopy. This discrepancy is particularly dramatic in some aquatic environments, in which plate counts and viable cells estimated by acridine orange staining can differ by four to six orders of magnitude, and in soil, in which 0.1–1% of bacteria are readily culturable on common media under standard conditions. It is now widely accepted that the application of standard microbiological methods for the recovery of microorganisms from the environment has had limited success in providing access to the true extent of microbial biodiversity. It follows that much of the extant microbial genetic diversity (collectively termed the metagenome) remains unexplored and unexploited, an issue of considerable relevance to a wider understanding of microbial communities. Incessant quest of man for newer and newer chemicals, drugs, and other resources from microbes that may have important bearing to the biotechnology industry has provided further impetus to this line of study. The recent development of technologies designed to access this wealth of genetic information through environmental nucleic acid extraction has provided a means of avoiding the limitations of culture-dependent genetic exploitation.

The visualization of microbial world was changed radically in 1985 by Carl Woese, whose work reflected that rRNA gene provides evolutionary chronometers (Woese 1987). A new branch of microbial ecology was created by Pace and his colleagues (Lane 1985; Stahl et al. 1985) by using direct analysis of 5S and 16S rRNA gene sequences from different environments. The analysis of these sequences was used to describe the diversity of microorganisms without culturing (Pace et al. 1986). The early studies relied on sequencing of reverse transcription-generated cDNA copies or direct sequencing of RNA. The development of PCR technology was the next technical breakthrough as by designing the appropriate primers virtually any gene and almost the entire gene could be amplified (Giovannoni et al. 1990). The new technique accelerated the discovery of diverse taxa as habitats across the earth could be surveyed (Barns et al. 1994; Eden et al. 1991). The application of PCR technology provided a view of microbial diversity that was not distorted by the culturing bias and revealed that the uncultured majority is unbelievably diverse and contains members that diverge deeply from the readily culturable minority. 16S rRNA gene sequences also provided an aid to culturing efforts in addition to providing a universal culture-independent means to assess the diversity. Culturing efforts have intensified recently due to nucleic acid probes labeled with fluorescent tags providing such an assay, facilitating quantitative assessment of enrichment and growth. Successes have included pure cultures of members of the SAR11 clade, now termed the genus *Pelagibacter* (Cho and Giovannoni 2004; Connon and Giovannoni 2002), which represents more than one-third of the prokaryotic cell types in the surface of the ocean but was known only by its 16S rRNA signature until 2002 (Morris et al. 2002). The Acidobacteria phylum (Janssen et al. 2002) is the corollary to SAR11 in terrestrial environments. Acidobacteria are abundant in soil, typically representing 20–30% of the 16S rRNA sequences amplified by PCR from soil DNA, but until recently only three members had been cultured (Barns et al. 1999). Given that many organisms will not be coaxed readily into pure culture, a critical advance is to extend the understanding of the uncultured world beyond cataloging 16S rRNA gene sequences, and microbiologists have striven to devise methods to analyze the physiology and ecology of these diverse, uncultured, hitherto unknown organisms.

Metagenomics

Among the methods designed to gain access to the physiology and genetics of uncultured microorganisms, metagenomics, or environmental genomics, the genome analysis of a population has emerged as a powerful centerpiece. Direct isolation of genomic DNA from an environment circumvents culturing the organisms under study, followed by cloning of genomic fragments into a culturable host that captures it for further study and preservation. With the feasibility of such a technique, numerous advances have been derived from sequence-based and functional analysis in samples from water and soil from diverse habitat and those associated with eukaryotic hosts.

Definition of Metagenomics

What is metagenomics? A review paper published in 2004 defines "metagenomics" as functional and sequence-based analysis of the collective microbial genomes contained in an environmental sample (Handelsman 2004). The report from the American Institute of Microbiology (2002) defines metagenomics as that "entails large-scale sequencing of pooled, community genomic material, with either random or targeted approaches, assembly of sequences into unique genomes or genome clusters, determination of variation in community gene and genome content or expression over space and time, and inference of global community activities, function, differentiation, and evolution from community genome data." Probably the oldest paper that used the term "metagenome" was published in 2000 (Woese 1987). However, the concept that organisms could be identified without cultivation by retrieving and sequencing them directly from nature is much older. Metagenomic approaches to capture microbial diversity in natural habitats have been employed by many researchers for years. The terms used to describe such methods include environmental DNA libraries, zoo libraries, soil DNA libraries, recombinant environmental libraries, whole genome treasures, community (environmental) genome analysis, whole genome shotgun sequencing, random community genomics, and probably others. Among them, metagenomics seems to be the most commonly used term to describe such studies and was used for the title of the first International Conference titled *Metagenomics 2003* organized by Dr. Christa Schleper in Darmstadt, Germany. Metagenomics combines the power of genomics, bioinformatics, and systems biology. Operationally, it is novel in that it involves study of the genomes of many organisms simultaneously.

Metagenomics is employed as a means of systematically investigating, classifying, and manipulating the entire genetic material isolated from environmental samples. This is a multistep process that relies on the efficiency of five main steps. The procedure consists of (a) the selection of an environmental niche, (b) the isolation of genetic material directly from an environmental sample, (c) manipulation of the genetic material, (d) library construction, and (e) the analysis of genetic material in the metagenomic library.

A sample is first collected that represents the environment under investigation because the biological diversity will be different in different environments. The second step of the procedure is the isolation of the DNA. As the samples may contain many different types of microorganisms, the cells are broken open using chemical methods such as alkaline conditions or physical methods such as sonication. Once the DNA from the cells is free, it must be separated from the rest of the materials in the sample. This is accomplished by taking advantage of the physical and chemical properties of DNA. Some methods of DNA isolation used include density centrifugation, affinity binding, and solubility/precipitation. Commercial kits are now available and are properly used for isolation of DNA from mixed samples (Lloyd-Jones and Hunter 2001).

Once the DNA is collected, it is manipulated so that it can be introduced in a chosen model organism. Genomic DNA is relatively large, so it is cut up into smaller fragments using enzymes called restriction endonucleases. These are special enzymes that cut DNA at a particular sequence of base pairs. Depending upon the

enzyme used, this results in the smaller, linear fragments of DNA carrying either staggered or flush ends. The fragments are then combined (ligated) with suitable vectors. Vectors are small units of DNA that can be transformed into cells where they can replicate and produce the proteins encoded on the introduced DNA using the machinery that the cells use to express normal genes. The vectors also contain a selectable marker. Selectable markers provide a growth advantage that the model organism would not normally have otherwise (such as resistance to a particular antibiotic) and are used to identify which cell contains vectors (transformed). The ones which do not contain vector (untransformed) are selected out.

The next step is to introduce the vectors with the metagenomic DNA fragments into the model organism, to generate metagenomic library. This allows the DNA from organisms that would not grow under laboratory conditions to be replicated, expressed, and studied. DNA inserted in the vector is transformed into cells of a model organism, typically *Escherichia coli*. Though the first choice always falls on *E. coli,* it is becoming clear that this bacterium cannot express all the genes. A search for an alternative host has to be kept in mind and should become a part of all such protocols. Transformation is the physical insertion of foreign DNA into a cell, followed by stable expression of proteins. It can be done by chemical, electrical, or biological methods. The method of transformation is determined based on the type of sample used and the required efficiency of the reaction. The metagenomic DNA in the vectors represents the entire DNA in the same sample initially, but the vectors are designed such that only one kind of DNA fragment from the sample will be maintained in each individual cell. The transformed cells are then grown on selective media so that only the cells carrying vectors will survive. Each group of cells that grows in a unit is called a colony. Each colony consists of many cloned cells that originated from one single cell. The population of cells containing all of the metagenomic DNA samples in vectors constitutes what is called metagenomic library. Each colony can be used to create a stock of cells for future study of a single fragment of the DNA from the environmental sample.

The clones can be then screened for expression of specific traits, such as enzyme activity or antibiotic production (function-based approach), or they can be sequenced randomly (sequence-based approach). The clones can also be screened for phylogenetic markers or "anchors," such as 16S rRNA and *recA*, or for other conserved genes by hybridization or multiplex PCR. This helps in taxonomic delineation of the source of the DNA (uncultured microbe). Each approach has strengths and limitations; together these approaches have enriched our understanding of the uncultured world, providing insight into groups of prokaryotes that are otherwise entirely unknown.

Metagenomic DNA Libraries

The basic steps of DNA library construction include generation of suitably sized DNA fragments, cloning of fragments into an appropriate vector, and screening for the gene of interest (Fig. 10.1). DNA fragmentation is a significant problem in constructing metagenomic libraries. Mainly vigorous extraction methods from environmental samples often result in excessive DNA shearing particularly when a higher yield is desired. An alternative approach uses blunt-end or T–A ligation to clone randomly sheared metagenomic fragments (Wilkinson et al. 2002).

Cosmid and bacterial artificial chromosome (BAC) libraries have been widely used for the construction of metagenomic libraries because of their ability to carry large DNA fragments (Beja 2004). Cloning such fragments of metagenomic DNA allows entire functional operons to be targeted with the possibility of recovering entire metabolic pathways. This approach has successfully been applied for the isolation of several multigenic pathways such as that responsible for the synthesis of the antibiotic violacein (Brady et al. 2001). Cosmid-sized (35–45 kbp) inserts in *E. coli* can also be stably maintained using fosmid vectors (Beja 2004).

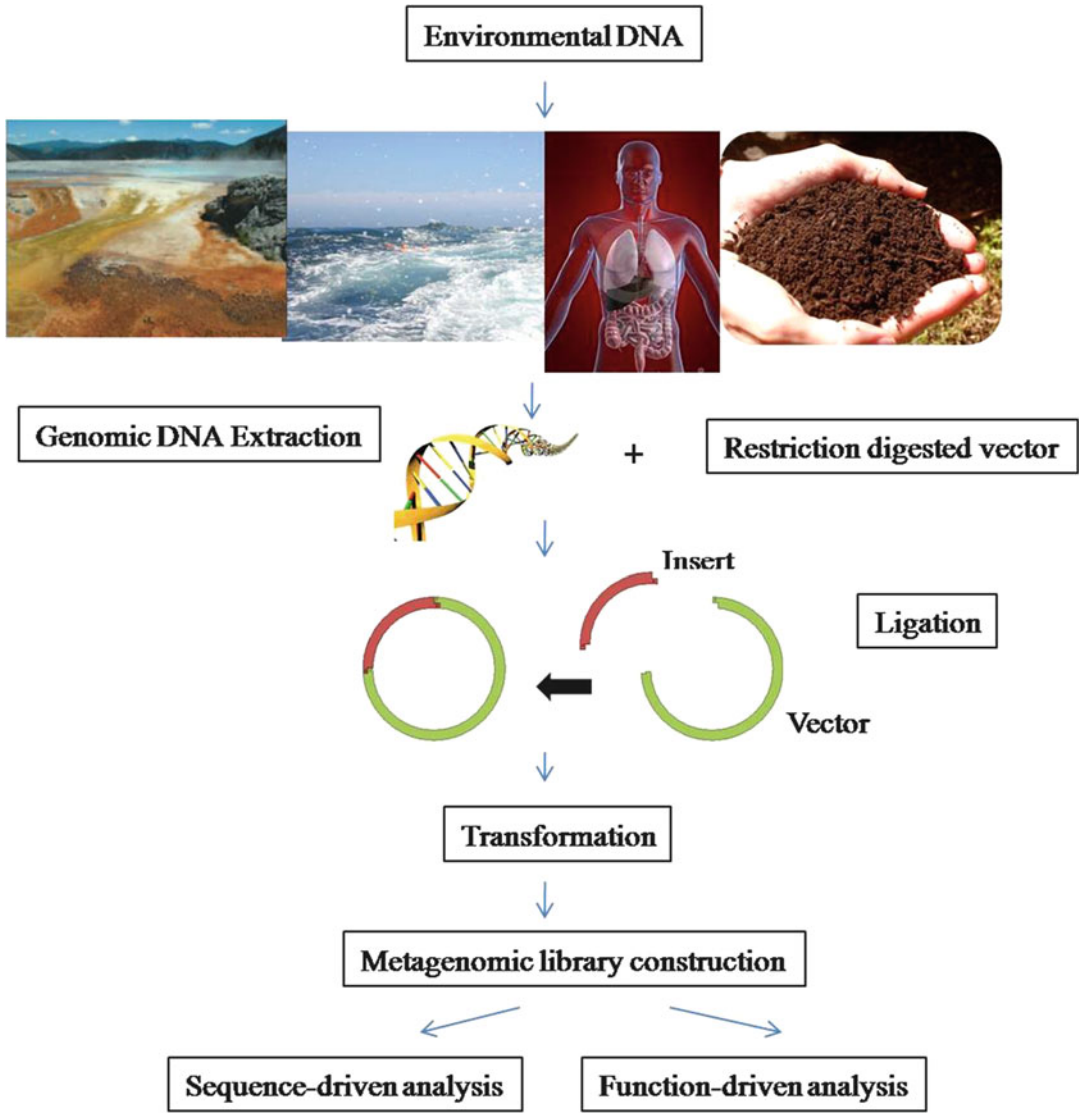

Fig. 10.1 Essential steps to explore and exploit the genomic diversity of microbial communities by metagenomics

The limitation of *E. coli* as a host for comprehensive mining of metagenomic samples is highlighted by the low number of positive clones obtained during a single round of screening (typically less than 0.01%). This suggests that without sample enrichment, the discovery of specific genes in a complex metagenome is technically challenging.

The assumption that expression in an *E. coli* host will not impose a further bias is largely untested. Although the *E. coli* transcriptional machinery is known to be relatively promiscuous in recognizing foreign expression signals, a bias in favor of *Firmicutes* genes has been established. The further development of host screening systems is therefore a fruitful approach for the more effective future exploitation of metagenomes.

Screening of Metagenomic Clones

Two strategies are generally used to screen and identify novel genes from metagenomic libraries: sequence-based analysis and function-based

Table 10.1 Sequence-driven versus function-driven analysis

Sequence-driven analysis	Function-driven analysis
Environmental sample	Extract metagenomic DNA
↓	↓
Construction of gene library using PCR	Clone into a vector
↓	↓
Sequencing	Introduce into a specific host
	↓
	Metagenomic library construction
	↓
	Functional screening for particular phenotype

analysis (Table 10.1). Both sequence- and function-based screenings have individual advantages and disadvantages, and they have been applied successfully to discover genes from metagenome (Table 10.2).

Sequence-Based Analysis

Sequence-based analysis can involve complete sequencing of cloned DNAs. As described earlier, clones containing phylogenetic anchors indicate the probable taxonomic group and identify the source of the DNA fragment. Alternatively, random sequencing can be conducted, and its function could be deduced by comparing it with the entries available in databases. Once a gene of interest is identified, phylogenetic anchors can be sought in the flanking DNA to provide a link of phylogeny with the functional gene. Identification of phylogenetic markers is a powerful approach guided by sequence analysis. It was first proposed by the DeLong group, which produced the first genomic sequence linked to a 16S rRNA gene of an uncultured archaeon (Stein et al. 1996). Subsequently, they identified an insert from seawater bacteria containing a 16S rRNA gene that affiliated with the Proteobacteria. The sequence of flanking DNA revealed a bacteriorhodopsin-like gene. Its gene product was shown to be an authentic photoreceptor, leading to the insight that bacteriorhodopsin genes are not limited to Archaea but are in fact abundant among the Proteobacteria of the ocean (Black et al. 1995; Bohlool and Brock 1974).

Sequencing random clones is an alternative to phylogenetic marker-driven approach, which has produced dramatic insights, especially when conducted on a massive scale. Sequence-based analysis can infer the distribution and redundancy of functions in a community, linkage of traits, genomic organization and detect horizontal gene transfer. The recent monumental sequencing efforts, which include reconstruction of the genomes of uncultured organisms in a community in the Sargasso Sea (Venter et al. 2004) and acid mine drainage (Tyson et al. 2004), illustrate the power of large-scale sequencing efforts to enrich our understanding of uncultured communities. These studies have made new linkages between phylogeny and function, indicated the surprising abundance of certain types of genes, and reconstructed the genomes of organisms that have not yet been cultured.

The power of this approach is likely to increase, as the collection of phylogenetic markers is growing. With the diversity of such markers, it became possible to assign more and more fragments of anonymous DNA to the organisms from which they could have likely been derived. There is limited utility of use of phylogenetic markers either as the initial identifiers of DNA fragments under study or as indicators of taxonomic affiliation for DNA fragments carrying genes of interest because their function is limited. Hence, the small number of available markers is a deterrent to provide reliable placement of the DNA source in the "Tree of Life" (Henne et al. 1999).

Function-Based Analysis

In function-based screening, clones expressing desired traits are selected from libraries, and

Table 10.2 Merits and demerits of sequence- and function-based analysis

Sequence-driven analysis	Function-driven analysis
Merits	
(1) Sequence-driven analysis overcomes the limitation of heterologous expression	(1) Function-driven analysis secures a complete form of gene or gene cluster required for desired traits
(2) Similar screening strategies can be used for different targets, for example, colony hybridization and PCR	(2) Completely novel genes can be recovered
Demerits	
(1) Sequence-driven analysis requires a database to analyze the DNA sequence, and it does not guarantee the acquisition of complete forms of gene	(1) Function-driven analysis must satisfy the expression conditions like transcription, translation, folding, and secretion
(2) Recovered genes are related to known genes	(2) It requires production of a functional gene product by the bacterial host

molecular and biochemical aspects of active clones are analyzed. Identification of clones that express a function is a powerful yet challenging approach to metagenomic analysis. Faithful transcription and translation of the gene or genes of interest and secretion of the gene product is required for its success, if the screen or assay requires it to be extracellular. Functional analysis has identified novel antibiotics (Courtois et al. 2003; Gillespie et al. 2002), antibiotic resistance genes (Diaz-Torres et al. 2003; Riesenfeld et al. 2004), $Na^+(Li^+)/H^+$ transporters (Majernik et al. 2001), and degradative enzymes (Healy et al. 1995; Henne et al. 1999, 2000a, b), to name a few. The power of the approach is that it does not require the gene(s) of interest be recognizable by sequence analysis, making it the only approach to metagenomics that has the potential to identify entirely new classes of genes for new or known functions. However, function-based screening has several limitations. This method requires expression of the function of interest in the host cell (e.g., *E. coli*), as well as clustering of all the genes required for the said function. Heterologous expression still remains a barrier in extracting the maximum information from functional metagenomics analyses.

When the functions of interest do not provide the basis for selection, high-throughput screens can substitute them. For example, active clones display a characteristic and easily distinguishable appearance on certain indicator media, even when plated at high density. Henne et al. (1999) detected clones that utilize 4-hydroxybutyrate in libraries of DNA derived from agricultural or river valley soil with the indicator dye tetrazolium chloride (Henne et al. 1999). Very rare lipolytic clones in the same libraries were detected by production of clear halos on media containing rhodamine and either triolein or tributyrin Henne et al. (1999). Catabolic enzyme genes can also be screened by substrate-induced gene expression (SIGEX).

High-throughput screening can also be done to identify compounds that induce the expression of genes under the control of a quorum-sensing promoter. This is a very powerful approach as the screen is intracellular, thus detecting that metagenomics DNA which is in the same cell as the sensor for quorum-sensing induction (Handelsman 2004). One of the very good examples of such a sensor comprises *lux*R promoter, which is induced by acylated homoserine lactones, linked to *gfp*. Promoter *lux*R resides on a plasmid in an *E. coli* strain that cannot induce quorum sensing. However, if an inducer of the luxR-mediated transcription of *gfp* is expressed from metagenomics DNA, the cell fluoresces and can be captured by fluorescence microscopy.

This sensor system can also detect inhibitors of quorum sensing, if acylated homoserine lactones are added to medium and fluorescence-activated cell sorting is set to collect the nonfluorescent cells. Arrays of genes have been identified from the metagenomics libraries of mid gut of the gypsy moth and microbiota of the soil.

The discovery of new biological motifs is dependent in part on functional analysis of metagenomic clones. Assignment of functions to numerous

"hypothetical proteins" in the databases has been done through functional screens of metagenomic libraries. To identify and overcome the barriers to heterologous gene expression and to detect rare clones efficiently in the immense libraries that represent all of the genomes in complex environments, further innovations in the techniques will be required. An emerging and powerful direction for metagenomic analysis is the use of functional anchors, which are the functional analogs of conventional phylogenetic anchors. Functional anchors define the functions that can be assessed rapidly in all of the clones in a library. When a collection of clones with a common function is assembled, they can be sequenced to identify phylogenetic anchors and genomic structure in the flanking DNA. Such an analysis can provide a slice of the metagenome that cuts across clones with a different selective tool, determining the diversity of genomes containing a particular function that can be expressed in the host carrying the library. Technological developments that promote functional expression and screening are bound to advance this new frontier of functional genomics (Handelsman 2004). Although function-driven screens usually result in identification of full-length genes (and therefore functional gene products), one limitation of this approach is its reliance on the expression of the cloned gene(s) and the functioning of the encoded protein in a foreign host.

Metagenomic studies have also been applied to environmental transcriptomes, where direct retrieval and analysis of microbial transcripts is done. In this approach, environmental mRNA is isolated. These mRNAs were then reverse transcribed, amplified with random primers, cloned, and functionally analyzed. This is a means of exploring functional gene expression within natural microbial communities without bias towards known sequences and provides a new approach for obtaining community specific variants of key functional genes (Pace et al. 1986).

Phage-display expression libraries provide a means for isolating DNA sequences by affinity selection of the surface-displayed expression product. This method is efficient and amenable to high-throughput screening, offering the potential to enrich even rare DNA sequences in the metagenome. Phage display was pioneered by George Smith in 1985, and it leads to full realization of the value of protein–ligand interaction. Phage display is used for many purposes. As a natural selection procedure, it is useful for generating targets for drug discovery (Benhar 2001; Trepel et al. 2002; Gnanasekar et al. 2004), epitope mapping (Matthews et al. 2002), and for screening antibodies (Prinz et al. 2004). Antibodies were one of the first proteins to be displayed on a phage surface (McCafferty et al. 1990), and the isolation of monoclonal antibodies has been one of the most successful applications of phage display to date (Hoogenboom et al. 1998).

Bacteriophages are estimated to total 1,031 virus particles (Brussow and Hendrix 2002). Due to the bacteria-killing activity of some phages, and to the diminishing power of antibiotics to treat disease, phage therapy is becoming commercially popular (Alisky et al. 1998; Miedzybrodzki et al. 2007; Capparelli et al. 2007; Easton 2009), and several important studies have been carried out on the possibility of developing phage as an alternative to antibiotics (Weber-Dabrowska et al. 2000; Wagenaar et al. 2005). Bacteriophages are used for phage display due to their natural ability to infect bacterial cells and because they can incorporate foreign DNA into their circular genome and transport them into a bacterial cell during infection (Smith and Petrenko 1997). However, phage display is limited by the expression capacity of the bacteriophage, a protein size with an upper limit of around 50 kDa (Crameri and Suter 1993). Filamentous phage display allows assembly in, and secretion from, an infected bacterium without compromising the host cell membrane (Mullen et al. 2006). *E. coli* cells infected with such bacteriophage become a factory for phage production, as the host machinery is commandeered to generate phage virions.

SIGEX

In 2005, Uchiyama et al. introduced a third type of function-based screen, which was termed as SIGEX. It has been developed for isolating novel catabolic genes from environmental metagenomes,

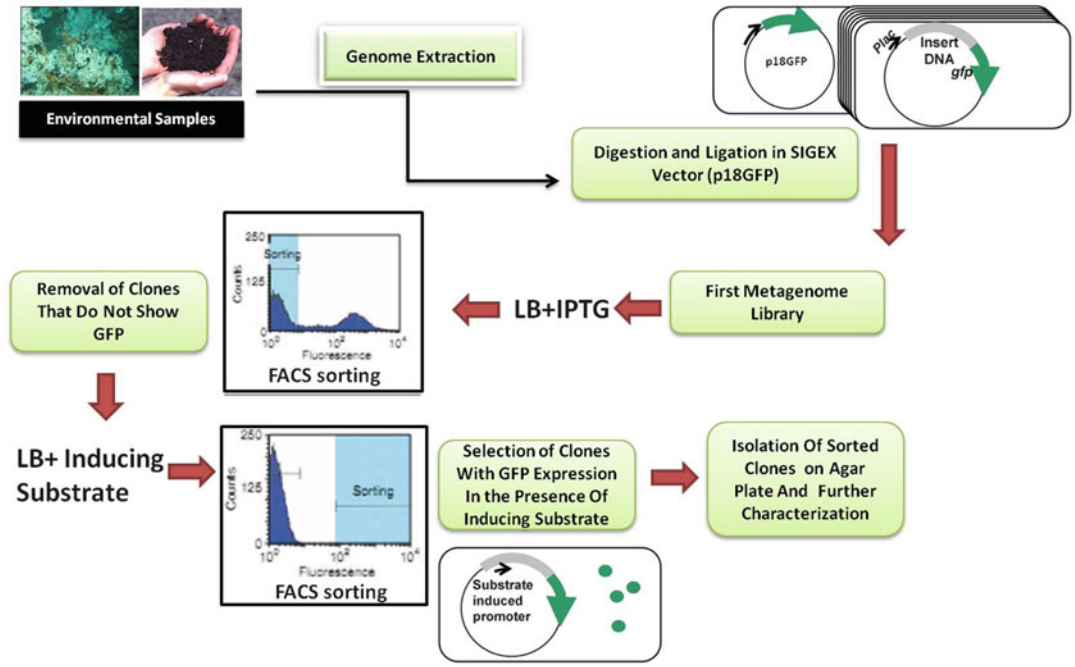

Fig. 10.2 Schematic representation of SIGEX

particularly genes that are difficult to obtain using conventional gene cloning methods. This high-throughput screening approach employs an operon-trap *gfp* expression vector in combination with fluorescence-activated cell sorting. In SIGEX, restriction enzyme-digested metagenome fragments are ligated into an operon-trap vector (e.g., p18GFP), and a library is constructed and grown in a liquid culture by transforming a cloning host (e.g., *E. coli*). The library is subjected to a substrate-dependent gene-induction assay, and positive cells are selected by detecting activity of a co-expressed marker (e.g., GFP) encoded in the vector. High-throughput screening is possible if FACS is used to select GFP-expressing cells (Fig. 10.2).

In this way, Uchiyama and Miyazaki isolated aromatic hydrocarbon-induced genes from a metagenomic library derived from groundwater (Uchiyama et al. 2005).

Limitations of this method are (1) only genes homologous to known genes can be obtained, (2) genes obtained may be partial, (3) many enzymes are difficult to be expressed in a heterologous host as an active form, (4) catabolic genes that are distant from a relevant transcriptional regulator cannot be obtained, and (5) it is sensitive to the orientation of genes with desired traits (Yun and Ryu 2005).

METREX

A similar type of screen, designated metabolite-regulated expression (METREX), has been published by Williamson et al. (2005). The goal of this study was to design and evaluate a rapid screen to identify metagenomic clones that produce biologically active small molecules. To identify clones of interest, biosensor detecting small, diffusible signal molecules that induce quorum sensing is placed inside the same cell as the vector harboring a metagenomic DNA fragment. If the clone produces a quorum-sensing inducer, the cell produces GFP and can be identified by fluorescence microscopy or captured by fluorescence activated cell sorting. METREX detected quorum-sensing inducers among metagenomic clones that a traditional overlay screen would not. One inducing clone carrying a LuxI

homologue has been identified. This way, later, Guan et al. (2007) identified a new structural class of quorum-sensing inducers from the mid-gut bacteria of gypsy moth larvae by employing this method. A monooxygenase homolog which produced small molecules that induced the activities of LuxR from *Vibrio fischeri* and CviR from *Chromobacterium violaceum* has been detected (Williamson et al. 2005; Guan et al. 2007).

PIGEX

In 2010, Uchiyama and Miyazaki introduced another screening based on inducer gene expression known as product-induced gene expression. It is a reporter assay-based screening method for enzymes, which was used to screen metagenomic library containing large number of clones. In this case, enzyme activities are detected by expression of *gfp*, which is triggered by product formation. In their study, transcriptional activator BenR was replaced upstream of *gfp*. *E. coli* cells harboring the benR-gfp cassette would fluoresce in the presence of a benzoate precursor compound if they expressed an enzyme capable of actively transforming the precursor into benzoate. This reporter assay system would allow the identification of desired enzymatic activities by linking product formation to reporter gene expression. Using this system, amidases were targeted which can convert benzamide to benzoate. Ninety-six thousand clones were screened, and 11 amidase genes were recovered from 143 fluorescent wells, 8 of which were homologous to known bacterial amidase genes, while 3 were novel genes (Uchiyama and Miyazaki 2010).

Sample Enrichment

In a metagenomic screening process (e.g., expression screening of metagenomic libraries), the target gene(s) represent a small proportion of the total nucleic acid fraction. Pre-enrichment of the sample thus provides an attractive means of enhancing the screening hit rate. The discovery of target genes can be significantly improved by applying one of several enrichment options, ranging from whole-cell enrichment to the selection and enrichment of target genes and genomes (Miller et al. 1999).

Culture enrichment on a selective medium favors the growth of target microorganisms. The inherent selection pressure can be based on nutritional, physical, or chemical criteria, although substrate utilization is most commonly employed. For example, a fourfold enrichment of cellulase genes in a small insert expression library was obtained by culture enrichment on carboxymethyl cellulose (Miller et al. 1999). Although culture enrichment will inevitably result in the loss of a large proportion of the microbial diversity by selecting fast-growing culturable species, this can be partially minimized by reducing the selection pressure to a mild level after a short period of stringent treatment.

Nucleic Acid Extraction and Enrichment Technology

Numerous community nucleic acid extraction methods have been developed. Mainly two principal strategies for the recovery of metagenomes are (a) cell recovery and (b) direct lysis. Extraction of total metagenomic DNA is essentially a compromise between the vigorous extraction required for the representation of all microbial genomes and the minimization of DNA shearing and the co-extraction of inhibiting contaminants as isolation of individual cell is rather a difficult process. Chemical lysis is a gentler method in comparison to mechanical bead beating, recovering higher molecular weight DNA. Chemical lysis can also select for certain taxa by exploiting their unique biochemical characteristics.

Total DNA extraction does not typically contain an even representation of the population's genome within a given environmental sample. The dominant organism overshadows the rare organisms. This could lead to bias towards conclusion and downstream manipulations such as PCR. This can be overcome by means of experimental normalization. Separation of genomes can be done by caesium chloride gradient centrifugation in

the presence of intercalating agent, such as bisbenzimide, for the buoyant density separation. The separation of genome is based on their %G+C content. Equal amount of each band on the gradient is combined to represent a normalized metagenome. Normalization can also be achieved by denaturing the fragmented genomic DNA first, then reannealing them under very stringent conditions (68°C for 12–36 h). The concept being, abundant ssDNA will anneal more rapidly as their number is higher than the rare dsDNA species. The ssDNA strands will be separated from dsDNA, resulting in enrichment of rarer sequences within the environmental sample.

Genome Enrichment Strategies

Many strategies are being employed for genome enrichment. One strategy is to target the active component of microbial populations. Such a strategy is aimed to tell us which species are functionally active in specific processes (Miller et al. 1999).

Stable-Isotope Probing (SIP)

Genome enrichment strategies can be used to target the active components of microbial populations. Stable-isotope probing (SIP) techniques involve the use of a stable-isotope-labeled substrate and density gradient centrifugal separation of the "heavier" DNA or RNA. After growing a mix of different microbial species on a simple ^{13}C-labeled substrate like ^{13}C-methanol, the ^{13}C DNA produced by methanol-utilizing species can be clearly separated from the ^{12}C DNA originating from species unable to utilize methanol. After DNA extraction from the growth medium, the newly formed ("heavy") ^{13}C DNA can be separated from the ("light") ^{12}C DNA by density-gradient centrifugation. The ^{13}C DNA can then be identified by comparing with DNA libraries and subsequently linked to the active microbial species. This method is called stable-isotope probing (DNA-SIP). Actively growing microorganisms can also be labeled with 5-bromo-2-deoxyuridine (BrdU), and the labeled DNA or RNA is separated by immunocapture or density gradient centrifugation (Urbach et al. 1999).

Suppressive Subtractive Hybridization (SSH)

This technique identifies the differences between different DNAs derived from microorganisms. Adaptors are ligated to the DNA populations, and subtractive hybridization is carried out to select for DNA fragments unique to each DNA sample. It is completely PCR based and eliminates the step of single-stranded tester cDNA purification by streptavidin–biotin or hydroxyapatite. For cDNA subtraction, the tester pool is divided in two fractions, and a different adaptor is ligated to each fraction. An excess of driver cDNA, without linkers (adapters), is denatured and hybridized with each tester (with linkers) cDNA pools (first hybridization). Both samples are mixed together with addition of more single-stranded driver (second hybridization). The resulting pool is a mixture of single stranded, double stranded with only one linker, double stranded like the original pools, and double stranded with both linkers corresponding to the tester-specific fragments. Filling the ends of the linkers allows creating templates to be amplified by PCR. Conception of the adaptors is such that the cDNA possessing the same kind of adaptor on both sides will form a hairpin preventing amplification. Only the ones possessing both linkers will be amplified exponentially. The resulting PCR product is enriched in tester-specific cDNAs. The products are cloned and characterized to confirm their specificity by cDNA microarray. This is a powerful tool for genome enrichment, but the complexity of metagenomes makes this detection difficult. Using multiple rounds of subtractive hybridization can increase the sensitivity of the process (Green et al. 2001).

Gene Enrichment Strategies

To selectively enrich for a specific target gene within a metagenome, a more practical approach would be to use one of several differential expression technologies that rely on the isolation of mRNA to target transcriptional differences in gene expression. For example, differential expression analysis (DEA) is a very effective tool for gene enrichment (Ochman et al. 1993).

Differential Expression Analysis (DEA)

In this approach, the expression of genes upregulated for the specific activity can be identified. DEA targets transcriptional differences in gene expression. Many variations in the basic concept exist today which include selective amplification via biotin and restriction-mediated enrichment (SABRE), integrated procedure for gene identification (IPGI), serial analysis of gene expression (SAGE), tandem arrayed ligation of expressed sequence tags (TALEST), and total gene expression analysis (TOGA). These techniques have been effectively applied for eukaryotic gene discovery, but none have been applied in a metagenomic context. Their high sensitivity and selectivity should enable small differences in expression of single copy genes to be detected (Futamata et al. 2001).

Gene Targeting

A number of PCR-based approaches designed to recover the flanking regions of a DNA fragment once its sequence is known have been reported (Futamata et al. 2001). Although suitable for use at a single-genome level, these methods are technically more difficult to apply at the metagenomic level due to the increased complexity of a multigenomic DNA sample. A desire to simplify this process led us to look at the development of other novel approaches.

One potentially powerful approach is based on in vitro hybridization of a genomic DNA sample with the target gene fragment acting as a probe. Genomic DNA is fragmented, and priming sites are introduced by ligation of adapters. The gene-specific PCR product is then used as a driver to selectively hybridize to full-length gene fragments in the DNA sample. These partially double-stranded full-length gene fragments can then be selectively separated from the single-stranded background (genomic DNA). To remove any residual background, the adapters are removed; because the full-length gene fragments are only partially double stranded, the priming sites will remain intact as the restriction enzyme can only act on double-stranded DNA within the priming site. The full-length gene can then be amplified. This method is particularly powerful for multigenomic cloning as the use of degenerate gene-specific primers on a metagenomic sample typically yields a population of target gene fragments. Genes coding for catabolic enzymes such as methane monooxygenase, ammonia monooxygenase, catechol dioxygenase, and phenol hydroxylase have been retrieved from the environment in order to gain insight into the genetic diversity of catabolic populations. It is currently expected that such genetic information could aid in understanding and advancing bioremediation (Daniel 2005).

However, as a tool for biocatalyst discovery, gene-specific PCR has two major drawbacks. First, the design of primers is dependent on existing sequence information and skews the search in favor of known sequence types. Functionally similar genes resulting from convergent evolution are not likely to be detected by a single gene-family-specific set of PCR primers. Second, only a fragment of a structural gene will typically be amplified by gene-specific PCR, requiring additional steps to access the full-length genes. Amplicons can be labeled as probes to identify the putative full-length gene(s) in conventional metagenomic libraries.

Environment Niche Selection

The microbial diversity of both cultured and uncultured microorganisms is a direct reflection of the environment from where they are derived.

Though highly variable, two major communities taken into consideration are soil and marine ones. To this is added another community that exists in association with or parasitic to eukaryotic organisms.

Metagenomics of Soil

Phylogenetic surveys of soil ecosystems have shown that the number of prokaryotic species found in a single sample exceeds that of known cultured members. Soil metagenomics, which comprises isolation of soil DNA and the production and screening of clone libraries, can provide a cultivation-independent assessment of the largely untapped genetic reservoir of soil microbial communities. This approach has already led to the identification of novel biomolecules. However, owing to the complexity and heterogeneity of the biotic and abiotic components of soil ecosystems, the construction and screening of soil-based libraries is difficult and challenging. This review describes how to construct complex libraries from soil samples and how to use these libraries to unravel functions of the resident microbial communities (Dunbar et al. 1999).

The recovery of microbial soil DNA that represents the resident microbial community and is suitable for cloning or PCR is still an important challenge, considering the diversity of microbial species (both cultured and uncultured), the large populations of soil microorganisms, and the complex soil matrix, which contains many compounds (such as humic acids) that bind to DNA and interfere with the enzymatic modification of DNA. By using universal primers for bacteria and archaea, phylogenetic surveys can be carried out by PCR amplification of 16S rRNA genes from soil DNA. These results have allowed cataloging and comparison of the microbial diversity in different soil habitats and the comparative analysis of changes in community structure owing to altered environmental factors (Ovreas 2000; Dunbar et al. 2002; Zhou et al. 2002; Yeager et al. 2004; Henne et al. 1999).

Construction of Soil DNA Libraries

Same steps are involved in constructing soil-based libraries, as the cloning of genomic DNA of individual microorganisms, that is, fragmentation of the soil DNA by restriction-enzyme digestion or mechanical shearing, insertion of DNA fragments into an appropriate vector system, and transformation of the recombinant vectors into a suitable host.

Construction of libraries from soil DNA and screening of these libraries by functional and sequence-based approaches was the major breakthrough in soil metagenomics. This technology paved the way for elucidating the functions of organisms in soil communities, for genomic analyses of uncultured soil microorganisms, and for the recovery of entirely novel natural products from soil microbial communities. In landmark studies, novel genes that encoded useful enzymes and antibiotics were recovered by direct cloning of soil DNA into plasmid, cosmid, or BAC vectors and screening of the generated libraries (Brady and Clardy 2000; Rondon et al. 2000; Ogram et al. 1987). The genes were identified using functional screens, and some having little homology to known genes were identified. This illustrates the enormous potential of the analysis of soil-based metagenomic libraries.

Several factors are important for the success of projects to generate and screen soil derived metagenomic libraries. For example, composition of the soil sample, collection and storage of the soil sample, the DNA extraction method used for high quality DNA recovery, representation of the isolated DNA from the microbial community present in the original sample, the host vector systems used for cloning, maintenance and screening and the screening strategy, all may affect the final outcome.

Many soil DNA extraction protocols have been published, and commercial soil DNA extraction kits are available (Lloyd-Jones and Hunter 2001). Two main methods are known for the DNA extraction from soil: direct lysis of cells contained in the sample matrix followed by separation of DNA from the matrix and cell debris, pioneered by Ogram et al. (Gabor et al. 2003), or

separation of the cells from the soil matrix followed by cell lysis. The crude DNA recovered by both methods is purified by standard procedures. The amounts of DNA isolated from different soil types using a selection of protocols range from less than 1 μg to approximately 500 μg of DNA per gram of soil (Brady and Clardy 2000). More DNA is recovered using the direct lysis approaches, perhaps because of the loss of biomass during separation. For example, Gabor et al. (Delong 2005) recorded a 10- to 100-fold reduction in the DNA yield using the cell separation approach compared with the direct lysis approach.

Metagenomics of Marine Microbial Community

Marine microbial communities were among the first microbial communities to be studied using cultivation-independent genomic approaches. Ocean-going genomic studies are now providing a more comprehensive description of the organisms and processes that shape microbial community structure, function, and dynamics in the sea. Through the insight of microbial community genomics, a more comprehensive view of uncultivated microbial species, genes and biochemical pathways, distributions, and naturally occurring genomic variability is being brought into sharper focus. Besides providing new perspectives on oceanic microbial communities, these new studies are now poised to reveal the fundamental principles that drive microbial ecological and evolutionary processes (Béjà et al. 2000).

Marine Microbial Case Studies

Several studies have used either large-insert DNA cloning techniques or whole genome shotgun (WGS) approaches or both to characterize marine microbial assemblages. The outcomes of these studies include the discovery of unsuspected mechanisms of light-driven energy generation in the ocean (Béja et al. 2002; Preston et al. 1996), a massive survey of the gene complement of Sargasso Sea microorganisms, and the characterization of metabolic pathways of methane-oxidizing archaea in deep-sea sediments (Hallam et al. 2004; Falkowski and de Vargas 2004; Kruger et al. 2003; Teeling et al. 2004).

Photobiology of Marine Picoplankton

Early forays into environmental genomics demonstrated the feasibility of obtaining informative genomic "snapshots" from uncultivated marine microorganisms (Beja et al. 2000; Nelson et al. 1999). Several surprising discoveries have come to light through recent surveys of genome fragments from bacterioplankton that were archived in BAC libraries. To identify genome fragments containing phylogenetic markers (for instance, rRNAs) and sequence flanking the genomic regions, a type of phylogenetically anchored chromosome walking (Kawarabayasi et al. 1999; Stein et al. 1996) has been one of the important approaches. A 130-kb BAC clone was isolated from an uncultivated SAR86 bacterium (Zhou et al. 2002) (an abundant component of α-proteobacteria in ocean surface waters) using this method. Sequencing of the 130-kb fragment revealed a new class of genes of the rhodopsin family (named proteorhodopsin) that had never before been observed in bacteria as a whole or in the ocean community. The new genes have similarities to the known genes called rhodopsins that capture light energy from the sun and couple this with carbon cycling in the ocean through non-chlorophyll based pathways. When the bacterial proteorhodopsin was expressed in *E. coli*, it functioned as a light-driven proton pump (Zhou et al. 2002). So this genomic survey of uncultivated marine bacteria led directly to the discovery of a new type of light-driven energy generation in oceanic bacteria. Later studies confirmed the presence of retinal-bound proteorhodopsin in the ocean and showed that optimized spectral "tuning" of bacterial rhodopsins matches depth-specific light availability. Shotgun sequencing from the Sargasso Sea has now verified both the abundance and diversity of this new class of photoproteins. The emerging understanding of proteorhodopsin taxonomic and environmental

distributions is providing new insights into gene and genome evolution in microbial populations (Tamas et al. 2002).

Sargasso Sea Metagenomics

The Sargasso Sea is a part of the North Atlantic Ocean, lying roughly between the West Indies and the Azores. Here, the heart of the Bermuda Triangle is covered by the strongest and most notorious sea on the planet – the Sargasso Sea so named because there is a kind of seaweed, which lazily floats over its entire expanse, called *Sargassum*. Environmental investigations in the nutrient-poor waters near Bermuda in the Sargasso Sea led to the discovery of 1,800 new species of bacteria and more than 1.2 million new genes. Scientists used a whole-genome shotgun sequencing technique to clone random DNA fragments from the many microbes present in the sample, generating a treasure-house of new information.

The Sargasso Sea is a complex and physically sprawling ecosystem. The phylogeny of the community members of this diversity has not been exhaustively surveyed, and the inputs and outputs are more difficult to quantify. Craig Venter, who pioneered the Human Genome Project, led a group of scientists who embarked on the largest metagenomics project to date, in which they sequenced over 1 billion bp and claim to have discovered 1.2 million new genes (Venter et al. 2004). They placed 794,061 genes in a conserved hypothetical protein group, which contains genes to which functions could not be confidently assigned. The next most abundant group contained 69,718 genes apparently involved in energy transduction. Among these were 782 rhodopsin-like photoreceptors, increasing the number of sequenced proteorhodopsin genes by almost 10-fold. Linkage of the rhodopsin genes to genes that provide phylogenetic affiliations, such as genes encoding subunits of RNA polymerase, indicated that the proteorhodopsins were distributed among taxa that were not previously known to contain light-harvesting functions, including the *Bacteroides* phylum (Venter et al. 2004).

An intriguing initial observation is that many of the genomes in the Sargasso Sea contain genes with similarity to those involved in phosphonate uptake or utilization of polyphosphates and pyrophosphates, which are present in this extremely phosphate-limited ecosystem. The phosphorus cycle is not well understood, and this collection of genomes provides a new route for discovery of the mechanisms of phosphorus acquisition and transformation. The resulting data represent the largest genomic data set for any community on earth and offer a first glimpse into the broad ensemble of adaptations underlying diversity in the oceans. Because microbes generally are not preserved in the fossil record, genomic studies provide the key to understanding how their biochemical pathways evolved (Vezzi et al. 2005).

Future studies will allow more insights into how these molecules function as well as opportunities for mining and screening the data for specific applications. The vast data set provides a foundation for many new studies by other researchers. Analyses using iron-sulfur proteins as benchmarks led one group, for example, to conclude that these data reflect diversity equal to that in all the currently available databases, suggesting that microbial diversity thus far has been vastly underestimated (Beja et al. 2000).

Practical Approach

Large-Insert Bacterial Artificial Chromosome and Fosmid Libraries

Several strategies for cultivation-independent genomic survey of marine microbial communities have been used. More recently, fosmids and bacterial artificial chromosomes (BACs) have been applied in genomic analyses of naturally occurring marine microorganisms (Béja et al. 2002; Preston et al. 1996). These vectors are particularly useful for stable, high-fidelity propagation of large DNA inserts. DNA fragments of up to 200 kb can be stably cloned in these vectors; therefore, one clone could represent 5–10% of the entire genome of a small bacterium. BAC clones prepared from microbial assemblage DNA can be easily screened

to identify and characterize the cloned gene fragments for functions or to evaluate phylogeny. The first example of characterization of a microorganism using this approach examined an abundant but uncultivated group of planktonic marine archaea (Stein et al. 1996). Several studies have expanded the characterization of uncultivated archaeal species using this general approach. BAC libraries are repositories of genomic material and can also serve as a valuable reference resource for further sequencing and in vitro biochemical experimentation.

Small-Insert Whole-Genome Shotgun Libraries

Another approach for cultivation-independent microbial genome characterization is a variant of whole-genome shotgun (WGS) sequencing. For pure bacterial cultures, the WGS approach has been important for obtaining complete genome sequences, including those of several marine bacteria and archaea. WGS sequencing has also been used to sequence microbial symbionts and, in one case, an extremely simple microbial biofilm assemblage (Chen et al. 2003). WGS sequencing relies on the preparation and end sequencing of small-DNA-insert libraries and subsequent sequence assembly *in silico*. The high-throughput nature of this approach makes it extremely attractive. Variations on this theme, using linker ligation and subsequent amplification techniques, have also been used to generate shotgun libraries from naturally occurring viral populations.

Till now, it seems that WGS approaches alone cannot adequately deconvolute whole genome sequences from complex microbial assemblages. As with the human genome sequencing effort, the most complete and reliable datasets will probably result from a combination of sequencing and analysis strategies. These will also probably include front-end cell purification strategies to reduce inherent complexity, followed by combined WGS and large-insert sequencing strategies. In combination, these approaches could enhance the accuracy, coverage, and reliability of genomics-based efforts to understand complex microbial communities. Nevertheless, WGS sequencing of microbial communities represents a powerful, even if expensive, approach for high-volume, single-pass gene survey and sampling (Delong 2005).

Viral Metagenomics

Viruses, most of which infect microorganisms, are the most abundant biological entities on the planet. Identifying and measuring the community dynamics of viruses in the environment is complicated more so, because less than 1% of microbial hosts have been cultivated. Also, there is no single gene that is common to all viral genomes, so total uncultured viral diversity cannot be monitored using approaches analogous to ribosomal DNA profiling. Metagenomic analyses of uncultured viral communities circumvent these limitations and can provide insights into the composition and structure of environmental viral communities.

Viral metagenomes mostly comprise novel sequences. Viral metagenomics, which also focuses on shotgun sequencing of metagenomes, gives insight into the vast and previously untapped diversity of viral communities in, for example, near-shore marine water samples (Breitbart et al. 2004), marine sediment sample (Breitbart et al. 2003), human fecal sample (Cann et al. 2005), and other fecal sample (Pedulla et al. 2003). When the marine sequences were first published, approximately 65% of them had no significant similarity to any sequence in the GenBank nonredundant database. Analyses 2 years later revealed that most of the viral sequences are still unique, despite the fact that the GenBank database has since more than doubled in size. Likewise, in the equine fecal metagenome, 68% of the sequences have no similarity to any sequence in GenBank (Pedulla et al. 2003). Genomic analyses of cultured phages also show that most of the open reading frames (ORFs) are novel (Wommack et al. 1999). By contrast, only about 10% of the sequences from environmental microbial metagenomes (Homann et al. 2004) and cultured microbial genomes (Tyson et al. 2004) are novel when analyzed in similar ways. These observations indicate that while much of the global microbial metagenome

is being sampled, the global viral metagenome is still relatively uncharacterized. That there is an even greater amount of biodiversity than that attributed to prokaryotic communities allows further hypotheses to be developed about the role of viral communities. Daubin and Ochman have gone on to hypothesize that the unique genes in microbial genomes in fact were acquired from the phage genomic pool (Wommack et al. 1999).

Isolation of viral community DNA representative for metagenomic analyses is complicated by the presence of free and cellular DNA. If the free DNA is not removed, the viral DNA signal will be lost. Similarly, at ~50 kb long, the average viral genome is about 50 times smaller than the average microbial genome (2.5 Mb), so any cellular contamination will overwhelm the viral signal (Homann et al. 2004). By an estimate, 200 L of seawater or 1 kg of solid material is a typical starting sample consisting of fecal, soil, and sediment samples suspended in osmotically neutral solutions before filtration. To separate the intact viral particles from the microorganisms and free DNA, a combination of differential filtration with tangential flow filters (TFF), DNase treatment, and density centrifugation in cesium chloride (CsCl) is used. Viruses sensitive to CsCl will disintegrate in this step, and very large or very small viruses will be lost in the filtration step. As assessed by pulse-field gel electrophoresis (Stahl et al. 1985) and epifluorescence microscopy, this protocol seems to capture most of the viral community. Once intact virions have been isolated, the viral DNA is extracted and cloned. Cloning of viral metagenomes representative is challenging, due to low DNA concentrations (~10–17 ng DNA per virion), modified DNA bases, for example, 5-(4-aminobutylaminomethyl) uracil and 5-methyl cytosine, and the presence of lethal viral genes such as holins and lysozymes. In order to circumvent these problems, it is necessary to concentrate virions from several hundred liters, in most water samples, to obtain enough DNA for cloning. The linker-amplified shotgun library (LASL) technique includes a PCR amplification step, which makes it possible to clone small amounts of DNA (1–100 ng). The PCR step also converts modified DNA into unmodified DNA. A shearing step disrupts lethal virus genes by shearing DNA into small fragments (~2 kb) and provides the random fragments necessary for cloning. It is possible to make representative metagenomic libraries, using this protocol, that contain viral fragments that are proportional to their concentrations in the original sample. LASLs typically contain millions of random clones. RNA and single-stranded DNA (ssDNA) viruses, however, cannot be cloned using this approach. Preliminary studies with random-primed reverse transcriptase and random-primed strand-displacement DNA polymerases indicate that these viral groups could be analyzed using metagenomic approaches (Homann et al. 2004).

Host-Associated Bacteria: Genomic Insights into Pathogenesis and Symbiosis

Pathogenesis

The amenability of host-associated microbes to physical separation makes them well suited to this approach, which is in contrast to organisms that reside in complex environmental communities. The first complete genome of an uncultured bacterium, the syphilis spirochaete, *Treponema pallidum*, was published in 1998 – a landmark in genome sequencing. Although the bacterial origin of syphilis was recognized a century ago, the infectious agent could not be isolated in continuous culture. The DNA that was used for sequencing the intracellular pathogen was obtained from the testes of infected rabbits by a series of lysis and centrifugation steps that eventually resulted in an essentially pure bacterial preparation. Sequence analysis immediately identified potential contributors to virulence and aided the development of DNA-based diagnostics (Piel 2004).

A year and a half of painstaking growth in coculture with human fibroblasts was necessary to obtain sufficient DNA to sequence the genome of the Whipple disease bacterium, *Tropheryma whipplei*. The sequence revealed deficiencies that indicated an explanation for the failure to propagate in culture. Based on these genomic insights, Renesto et al. (2003) used a standard

tissue-culture medium, supplemented with amino acids that were implicated by the sequence analysis, to successfully cultivate *T. whipplei* in the absence of host cells, shortening their doubling time by an order of magnitude. This is one of many cases in which DNA sequence information has been used to improve culture techniques, diagnostics, and therapies for fastidious organisms (Wommack et al. 1999).

Symbiosis

Many bacterial symbionts that have highly specialized and ancient relationships with their hosts do not grow readily in culture. Many of them live in specialized structures, often in pure or highly enriched state, in host tissues, making them ideal candidates for metagenomic analysis because the bacteria can be separated readily from host tissue and other microorganisms. This type of analysis has been conducted with *Cenarchaeum symbiosum*, a symbiont of a marine sponge, a *Pseudomonas*-like bacterium that is a symbiont of *Paederus* beetles, *Buchnera aphidicola*, an obligate symbiont of aphids, the Actinobacterium, *Tropheryma whipplei*, the causal agent of the rare chronic infection of the intestinal wall, and the *Proteobacterium* symbiont of the deep-sea tube worm *Riftia pachyptila*. These systems provide good models for metagenomic analysis of more complex communities and thus warrant further attention in this review, although the term metagenomics typically connotes the study of multispecies communities (Handelsman 2004).

Tube Worm Symbiosis: *Proteobacterium*

Riftia pachyptila, the deep-sea tubeworm, lives 2,600 m below the ocean surface, near the thermal vents that are rich in sulfide and reach temperatures near 400 °C. The tube worm does not have a mouth or digestive tract, and therefore it is entirely dependent on its symbiotic bacteria, which provide the worm with food. The bacteria live in the trophosome, a specialized feeding sac inside the worm (Piel 2004). The bacteria and trophosome constitute more than half of the animal's body mass. The bacteria oxidize hydrogen sulfide, thereby producing the energy required to fix carbon from CO_2, providing sugars and amino acids (predominantly as glutamate) that nourish the worm (Piel 2004). The worm contributes to the symbiosis by collecting hydrogen sulfide, oxygen, and carbon dioxide and transporting them to the bacteria.

The bacterium is a member of the γ-Proteobacteria, as identified by 16S rRNA gene sequence. The bacteria have not been grown in pure culture in laboratory media, but they provide an excellent substrate for metagenomics because they reach high population density in the trophosome and exist there as a single species. Hughes et al. (1997) isolated DNA from the bacterial symbiont and constructed fosmid libraries that were used to understand the physiology of the bacteria. Robinson et al. (1998) identified a gene with similarity to ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) from the same fosmid library. All the residues associated with the active site are conserved in the protein sequence deduced from the DNA sequence, and it has highest similarity with the RubisCO from *Rhodospirillum rubrum*. The characterization of this gene lends further support to the premise that the chemoautotrophic bacterial symbiont in *R. pachyptila* fixes carbon for its host. The libraries were also screened for two-component regulators with a labeled histidine kinase gene as a probe. They identified a two-component system whose components complemented an *env*Z and a *pho*R *cre*C double mutant, respectively.

The discovery of a functional *env*Z homologue indicates that the symbiont carries a response regulator that is typical of γ-Proteobacteria, although the signals eliciting responses from these proteins have not yet been functionally identified (Handelsman 2004).

Gut Microbiome

Our body surfaces are home to microbial communities whose aggregate membership outnumbers our human somatic and germ cells by at least an order of magnitude. The majority of microbes

that reside in the gut, have a profound influence on human physiology and nutrition, and are crucial for human life. Furthermore, the gut microbes contribute to energy harvest from food, and changes of gut microbiome may be associated with bowel disease or obesity. They synthesize essential amino acids and vitamins and process components of otherwise indigestible contributions to our diet such as plant polysaccharides (Qin et al. 2010).

To understand and exploit the impact of the gut microbes on human health and well-being, it is necessary to decipher the content, diversity, and functioning of the microbial gut community. 16S ribosomal RNA gene sequence-based methods revealed that two bacterial divisions, Bacteroidetes and the Firmicutes, constitute over 90% of the known phylogenetic categories and dominate the distal gut microbiota. Metagenomic sequencing represents a powerful alternative to rRNA sequencing for analyzing complex microbial communities (Gill et al. 2006).

Illumina-based metagenomic sequencing has been used by Qin et al. (2010), where assembling and characterization of 3.3 million nonredundant microbial genes, derived from 576.7 gigabases of sequence, have been done from fecal samples of 124 European individuals. The gene set, ~150 times larger than the human gene complement, contains an overwhelming majority of the prevalent microbial genes of the cohort and includes a large proportion of the prevalent human intestinal microbial genes. The genes are largely shared among individuals of the cohort. Over 99% of the genes are bacterial, indicating that the entire cohort harbors between 1,000 and 1,500 prevalent bacterial species and each individual at least 160 such species, which are also largely shared. It has been found that gut microbiome has significantly enriched metabolism of glycans, amino acids, and xenobiotics; methanogenesis; and 2-methyl-erythritol 4-phosphate pathway-mediated biosynthesis of vitamins and isoprenoids.

The application of metagenomics to the medical field has led to a highly productive integration of clinical, experimental, and environmental microbiology. The functional roles played by human microbiota are closely looked into either through animal models or studies of human populations. Of particular interest is the fact that several human diseases have been linked to alterations in the composition and dynamics of human microbiota. The inputs from human microbiome and these based on human gene expression and variability and their application are a subject of great scientific challenge and interest (Frank et al. 2011).

Biogeochemical Cycles

Metagenomics provides an important insight into the community-wide assessment of metabolic and biogeochemical function. Analysis of specific functions across all members of a community can generate integrated models about how organisms share the workload of maintaining the nutrient and energy budgets of the community in an environment. The models can then be tested with genetic and biochemical approaches. An example of such an analysis is the nearly complete sequencing of the metagenome of a community in acid drainage of the Richmond mine. This mine is known to be representing one of the most extreme environments on Earth. The microbial community forms a pink biofilm that floats on the surface of the mine water. The drainage water below the biofilm has a pH between 0 and 1 and high levels of Fe, Zn, Cu, and As. The temperature around the biofilm water is 42 °C and is microaerophilic, having no source of carbon or nitrogen other than the gaseous forms in the air. Few of the most prominent bacterial members of the community are *Leptospirillum*, *Sulfobacillus*, and sometimes *Acidimicrobium*, one archaeal species, *Ferroplasma acidarmanus*, and other members of its group, the Thermoplasmatales. The mine is rich in sulfide minerals, including pyrite (FeS_2), which is dissolved as a result of oxidation, and is catalyzed by microbial activity. Tyson et al. (2004) were able to clone total DNA, because of its simple community structure, and sequenced most of the community with high coverage. The G+C content of the genomes of the dominant taxa in the mine differs substantially, thus providing the good indicator of its source. Sequence alignment

of 16S rRNA and tRNA synthetase genes confirmed the organismal origins of the clones. Nearly complete genomes of *Leptospirillum* group II and *Ferroplasma* type II were reconstructed, and substantial sequence information for the other community members could be obtained (Tyson et al. 2004).

The metagenomic sequence substantiated a number of significant hypotheses. First, it appears that *Leptospirillum* group III contains genes with similarity to those known to be involved in nitrogen fixation, suggesting that it provides the community with fixed nitrogen. This was a surprise because the previous supposition was that a numerically dominant member of the community, such as *Leptospirillum* group II, would be responsible for nitrogen fixation. However, no genes for nitrogen fixation were found in the *Leptospirillum* group II genome, leading to the suggestion that the group III organism is a keystone species that has a low numerical representation but provides a service that is essential to community function. *Ferroplasma* type I and II genomes contain no genes associated with nitrogen fixation but contain many transporters that indicate that they likely import amino acids and other nitrogenous compounds from the environment.

Energy appears to be generated from iron oxidation by both *Ferroplasma* and *Leptospirillum* spp. The genomes of both groups contain electron transport chains, but they differ significantly. The genomes of *Leptospirillum* group II and III contain putative cytochromes that typically have a high affinity for oxygen. The cytochromes may play a role in energy transduction as well as in maintaining low oxygen tension, thereby protecting the oxygen-sensitive nitrogenase complex. All of the genomes in the acid mine drainage are rich in genes associated with removing potentially toxic elements from the cell. Proton efflux systems are likely to be responsible for maintaining the nearly neutral intracellular pH, and metal resistance determinants pump metals out of the cells, maintaining nontoxic environment in the interior of the cells (Tyson et al. 2004).

The acid mine drainage community provides a model for the analysis of other communities. Determining the origin of DNA fragments and assigning functions may be more difficult for communities that are phylogenetically or physiologically more complex and variable, but the approach will be generally useful for all communities (Tyson et al. 2004).

Metagenomics and Industrial Application

Besides the vast academic output, one of the major interests in metagenome analysis is its immense economic potential. Different industries have different motivations to explore the enormous resource that constitute the uncultivated microbial diversity. Currently, there is a global political drive to promote white (industrial) biotechnology as a central feature of the sustainable economic future of modern industrialized societies. This requires the development of novel enzymes, processes, products, and applications. Metagenomics promises to provide new molecules with diverse functions, but ultimately, expression systems are required for any new enzymes and bioactive molecules to become an economic success.

Metagenomics (Barns et al. 1999) has the potential to substantially impact industrial production. The dimensions of the enormous biological and molecular diversity, as shown by Torsvik and Venter (Lorenz and Eck 2005) and their coworkers, are truly astonishing. A pristine soil sample might contain in the order of 10^4 different bacterial species. More than one million novel open reading frames, many of which may encode putative enzymes, could be identified in a single effort such that sampled marine prokaryotic plankton retrieved from the Sargasso Sea.

Different industries are interested in exploiting the resource of uncultivated microorganisms that has been identified through large-scale environmental genomics for several reasons detailed below.

The Ideal Biocatalyst

For any industrial application, enzymes need to function sufficiently well according to several

application-specific performance parameters. With the exception of yeasts and filamentous fungi, access to novel enzymes and biocatalysts has largely been limited by the comparatively small number of cultivable bacteria.

Novelty

For industries that produce bulk commodities such as high-performance detergents, a single enzyme backbone with superior functionality that has an entirely new sequence would be useful to avoid infringing competitors' intellectual property rights.

Maximum Diversity

The pharmaceutical and supporting fine-chemicals industries often seek entire sets of multiple, diverse biocatalysts to build in-house toolboxes for biotransformations (Lorenz and Eck 2005). These toolboxes need to be made rapidly accessible to meet the strict timelines of a biosynthetic-feasibility evaluation in competition with traditional synthetic chemistry.

Elusive Metabolites

Many pharmacologically active secondary metabolites are produced by bacteria that live in complex consortia or by bacteria that inhabit niches that are difficult to reconstitute in vitro. So although there are reports on how to circumvent this general problem of microbial cultivation either by mimicking natural habitats (Lorenz and Eck 2005) or by allowing for interspecies communication after single cell microencapsulation (Kaeberlein et al. 2002), the cloning and heterologous expression of biosynthetic genes that encode secondary metabolites (usually present as gene clusters) is the most straightforward and reproducible method of accessing their biosynthetic potential.

White Biotechnology

"Industrial" or white biotechnology is currently a buzzword in the European biobusiness community. The term was coined in 2003 by the European Association for Bioindustries (EuropaBio), based on a case study report, and it denotes all industrially harnessed bio-based processes that are not covered by the red biotechnology (medical) or green biotechnology (plant) labels. White biotechnology has its roots in ancient human history, and its products are increasingly a part of everyday life, from vitamins, medicines, biofuel, and bioplastics to enzymes in detergents or dairy and bakery products. It is the belief of industrial promoters, analysts, and policy makers that white biotechnology has the potential to impact industrial production processes on a global scale. The main long-term applications of white biotechnology will be focused on replacing fossil fuels with renewable resources (biomass conversion), replacing conventional processes with bioprocesses (including metabolic engineering), and creating new high-value bioproducts, including nutraceuticals, performance chemicals, and bioactives. While the possibilities are immense, some success stories are already being cited (Zengler et al. 2002).

Screening Enzymes for Industrial Use

Diversa, the largest and most prominent specialist biotech company for the commercialization of metagenome technologies, has described several approaches to access "uncultivable" microorganisms. Applying a classical growth-selection-based expression strategy, diverse environmental libraries were constructed in *E. coli* using phage λ or Bac vectors. After growth in media containing nitriles as the sole nitrogen source, more than 100 new and diverse nitrilase genes were recovered (Zengler et al. 2002). The resulting enzyme library is marketed to serve the fine-chemical and pharmaceutical industries (Robertson et al. 2004).

In addition to new technologies to amplify DNA from limited resources using random primers and strand-displacing DNA polymerase from phage Φ29 (DeSantis et al. 2002), a strategy promoted by Lucigen (Middleton, Wisconsin, USA), it is clear that current mass-sequencing efforts in several laboratories will facilitate the *in silico* identification of open reading frames that might encode potentially useful enzymes (Zhou et al. 2002).

Once new genes are cloned and screened for activity, the main stumbling block is the expression of pure protein in sufficient amounts at reasonable costs. A cheap and efficient enzyme, usually produced in efficient expression systems like bacilli or filamentous fungi, is a key factor for success, particularly when the enzyme functions as part of the final (bulk) product such as in detergents. In the fine-chemical industry, there might be a similar consideration for bulk product synthesis. Particularly in the pharmaceutical industry, the time taken for a target compound to come to the market is decisive, and in these applications, it might be even more important for a company to have a large collection of biochemically diverse catalysts, even if these molecules are not expressed in large amounts.

There is ample demand for novel enzymes and biocatalysts, and metagenomics is currently thought to be one of the most likely technologies to provide the candidate molecules required (Detter et al. 2002). The diversity of potential substrates for enzymatic transformations in the fine-chemical industry and the short time that is usually available to propose viable synthetic routes (particularly for the pharmaceutical industry) make it useful to produce pre-characterized enzyme libraries using generic substrates, before screening for a specific enzyme that is required for biotransformation of a particular substrate of interest (Lorenz et al. 2002).

Since the inception of two pioneering commercial metagenomics ventures in the late 1990s (Recombinant Biocatalysis Ltd of La Jolla and TerraGen Discovery Inc. of Vancouver), these technologies have been taken up by several of the biotechnology giants and have been the focal area of several start-up companies. Recombinant Biocatalysis Ltd, now Diversa Corporation, is the acknowledged leader in the field with impressive lists of libraries derived from global biotopes and of cloned enzymes in a range of enzyme classes. Several other smaller biotechnology companies appear to be competing in the same market sector, and others are obviously knocking at the door. The relatively small size of the industrial enzyme market compared with the pharmaceuticals market suggests that a switch in product focus might not be unexpected. Although the authors are unaware of any successfully commercialized therapeutics derived from metagenomic screening programs, the normal timelines for the identification, development, evaluation, and approval of products for the pharmaceuticals market are longer than the existence of metagenomics as a research field (Vakhlu et al. 2008).

Next-Generation Sequencing Approaches to Metagenomics

Next-generation sequencing approaches enable us to gather many more times sequence data than was possible a few years ago. The first next-generation high-throughput sequencing technology, the 454 GS20 pyrosequencing platform, which was developed by Roche, became available in 2005. The GS20 platform has now been replaced by GSFLX platform. Illumina released Solexa GA in early 2007, and more recently, SOLiD and Heliscope were released by Applied Biosystems and Helicos. Rapid advances in sequencing technology are fueling a vast increase in the number and scope of metagenomics projects. Most metagenome sequencing projects so far have been based on Sanger or Roche-454 sequencing, as only these technologies provide long enough reads, while Illumina sequencing has not usually been considered suitable for metagenomic studies due to a short read length of only 35 bp. However, now that reads of length 75 bp can be sequenced in pairs, Illumina sequencing has become a viable option for metagenome studies as well. A new software MEGAN has been evolved for metagenome analysis that allows one to process sequencing reads in pairs and makes assignments of such

reads based on the combined bit-scores of their matches to reference sequences. By using next-generation sequence data in metagenomics experiments, a wide range of new analyses are possible. Metagenomic study has an increasingly powerful partner in the next-generation sequence technology, and this partnership is likely to get more productive as softwares and hardwares mature (MacLean et al. 2009; Mitra et al. 2010).

Metatranscriptomics

As metagenomic DNA-based analyses cannot differentiate between expressed and non-expressed genes, it is unable to reflect the actual metabolic activity. To identify RNA-based regulation and expressed biological signatures in complex ecosystems, sequencing and characterization of metatranscriptomics have been employed. There are several difficulties associated with the processing of environmental RNA sample mainly due to the recovery and enrichment of high quality mRNA by the removal of other RNA species. Short half-lives of mRNA and low yield of cDNA include technical challenges in the isolation procedure. Initially, metatranscriptomics had been limited to the microarray and high-density array technological analysis of mRNA derived cDNA cloning. Detection sensitivity of microarray is not equal for all imprinted sequences as it can give information about those sequences for which it was designed and the result is also dependent on chosen hybridization condition. Transcript cloning avoids some of the problems, but it introduces other biases associated with cloning system and library construction. Substantial progress for the efficient analysis of more complex expression profiles has become available with the development of next-generation sequencing technologies like Roche's 454, Illumina's SOLEXA, and ABI'S SOLiD (Schloss and Handelsman 2003; Warnecke and Hess 2009).

In 2006, Leininger and colleagues were the first employing pyrosequencing to unravel active genes of soil microbial communities. This study has revealed that archaeal transcripts of key enzyme for ammonia oxidation were several magnitudes higher in soil than the bacterial version of it. In 2008, Friaz-Lopez et al. produced ≥50 Mbp by 454 pyrosequencing – still using first generation of this technology (MacLean et al. 2009). Gilbert et al. followed shortly afterwards with ≥300 Mbp of sequence data using second generation called GS-FLX (Gilbert et al. 2008). Other metatranscriptomic studies employing direct sequencing of cDNA have targeted the ocean surface water from North Pacific subtropic gyre, a phytoplankton bloom in the Western English Channel, coastal waters of a fjord close to Bergen, Norway, etc. All these studies demonstrated successful application of high-throughput sequencing technologies to exploit unknown transcripts that have been isolated directly from complex environment (Simon and Daniel 2011).

Metaproteomics

With the availability of metagenomic sequences, it is now possible to apply postgenomic techniques – particularly proteomics – to complex microbial communities as well. In 2004, Wilmes and Bond coined the term "metaproteomics" as a shotgun for large-scale characterization of the entire protein complement of environmental microbiota at a given time point. Protein expression is a reflection of specific microbial communities. Elucidation of metaproteomic expression is supposed to be central to functional studies of microbial consortia. In this study, an outer membrane protein and an acetyl coenzyme A acyl transferase were produced by a microbial community derived from activated sludge. These are highly expressed and putatively originated from an unculturable polyphosphate-accumulating *Rhodocyclus* strain dominating in the activated sludge (Mitra et al. 2010; Simon and Daniel 2011).

The landmark metaproteomic investigation successfully combined "shotgun" MS proteomics with the community genome analyses. This study analyzed the protein complement of a low-complexity natural biofilm, growing inside the Richmond Mine at Iron Mountain, CA (USA), having very low pH (0.8), a temperature of 42°C, and high level of heavy metals (Detter et al. 2002).

It was found that sampled biofilm was dominated by *Leptospirillum* group II along with the presence of *Leptospirillum* group III, *Sulfobacillus*, and Archaea related to *Ferroplasma acidarmanus.* Using the genome dataset, a total of 12,148 protein sequences were constructed. Challenges for metaproteomic analysis include uneven species distribution, large heterogeneity within microbial community, and broad range of protein expression levels within microorganisms. Despite these hurdles, metaproteomics will provide a new dimension of environmental catalysis (Simon and Daniel 2011; Leininger et al. 2006).

Metagenomics is a burgeoning field with new challenges encountered at every step in each instance. The gamut of challenges runs from inefficiencies in sampling, DNA extraction methods, and construction of libraries to inadequacies in data analysis and visualization tools. Added to this are limited computational power and data storage constraints due to the huge amounts of genomic data flooding in from initiatives worldwide. Some of these intricacies will have to be kept in mind, while garnering the full advantages of the metagenomic analyses, both from academic and application point of view (Leininger et al. 2006).

Low Abundance Species Overlooked

The high complexity environment of the Sargasso Sea comprising ~1,800 different species was daunting in terms of metagenome assembly and analysis. Many current assembly software are befuddled by the large numbers of complex, polymorphic metagenomic data, as are the annotation software, which are designed for use on "closed" (completely assembled) microbial genomes. Assembly is also hampered by shallow sequence coverage resulting from failures to sample uniformly, particularly in high-complexity environments where relative abundance of individual species varies. Most of the sequences obtained may be from the most predominant species in the environment, while sequences from low-abundance species may go undetected. These low-abundance species may well play a critical role in the ecophysiology of the habitat (Leininger et al. 2006; Wilmes and Bond 2004).

Lack of Reference Genomes

Sometimes, assembly can be assisted by the availability of a preexisting reference genome that can serve as a blueprint for piecing environmental genomic data together. Of course, such reference genomes are presently only available for a subset of cultured species, so assembling genomes of more divergent or novel species is not always an easy task. Finally, intraspecies heterogeneity or polymorphisms, or high levels of sequence conservation between phylogenetically unrelated genomes, all can confound the assembly software and result in false or chimeric assemblies (http://web.camera.calit2.net/cameraweb).

Standardizing Metadata

Metadata refers to the temporal, spatial, and physicochemical data associated with the sampling site from which organisms were derived for the metagenomics study. Typical examples are time, date, latitude, longitude, temperature, pH, salinity, etc. The purpose of making such metadata available is to enable correlation of deciphered ecology with the environmental conditions that may favor one population structure over another. Presently, there are no established standards for submission of metadata, and a Genomics Standards Consortium is involved in soliciting opinions from the research community to define a minimal set of metadata required for every genomic and metagenomic project (http://web.camera.calit2.net/cameraweb).

Conclusions

It is perhaps too early to state that metagenomic gene discovery is a technology that has "come of age." New approaches and technological innovations are pouring in on a regular basis and many

of the technical difficulties still waiting to be fully resolved. However, there can be little doubt that the field of metagenomics gene discovery offers enormous scope and potential for both fundamental microbiology and biotechnological development.

The genomes of the total microbiota found in nature contain huge untapped genetic information, which is accessible by metagenomic approaches. Yet the surface of this resource has been barely scratched as far as microbial genomes are concerned. The awareness of the real scope of microbial genome diversity and growing interest in biotechnological application of microbial products as pharmaceuticals, bioactive catalysts, biomaterials, and so forth must prompt the development of new research techniques for the direct and indirect acquisition of these genomes. Although there is unarguably a great need for future leaps in techniques for isolating and culturing novel microorganisms, the recent development of metagenomics, a field that effectively circumvents the microbial isolation and culturing, has been a major breakthrough.

References

Alisky J, Iczkowski K, Rapoport A, Troitsky N (1998) Bacteriophages show promise as antimicrobial agents. J Infect 36:5–15

Arima K, Wooly J (2008) Metagenomics. In: Xu Y, Gogarten JP (eds) Computational methods for understanding Bacterial and Archeal Genomes. Imperial College Press, London, pp 345–466

Barns SM, Fundyga RE, Jeffries MW, Pace NR (1994) Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci USA 91:1609–1613

Barns SM, Takala SL, Kuske CR (1999) Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. Appl Environ Microbiol 65:1731–1737

Beja O (2004) To BAC or not to BAC: marine ecogenomics. Curr Opin Biotechnol 15:187–190

Beja O et al (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902–1906

Béjà O et al (2000) Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environ Microbiol 2:516–529

Béja O et al (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature 415:630–633

Benhar I, Eshkenazi I, Neufeld T, Opatowsky J, Shaky S, Rishpon J (2001) Recombinant single chain antibodies in bioelectrochemical sensors. Talanta 55:899–907

Black C, Fyfe JAM, Davies JK (1995) A promoter associated with the neisserial repeat can be used to transcribe the uvrB gene from Neisseria gonorrhoeae. J Bacteriol 177:1952–1958

Bohlool BB, Brock TD (1974) Immunofluorescence approach to the study of the ecology of Thermoplasma acidophilum in coal refuse material. Appl Microbiol 28:11–16

Brady SF, Clardy J (2000) Long-chain N-acyl amino acid antibiotics isolated from heterologously expressed environmental DNA. J Am Chem Soc 122:12903–12904

Brady SF et al (2001) Cloning and heterologous expression of a natural product biosynthetic gene cluster from cDNA. Org Lett 3:1981–1984

Breitbart M et al (2003) Metagenomic analyses of an uncultured viral community from human feces. J Bacteriol 185:6220–6223

Breitbart M et al (2004) Diversity and population structure of a nearshore marine sediment viral community. Proc Biol Sci 271:565–574

Brussow H, Hendrix RW (2002) Phage genomics: small is beautiful. Cell 108:13–16

CAMERA (Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis) (2010) v1.3.2.32site. http://web.camera.calit2.net/cameraweb

Cann A, Fandrich S, Heaphy S (2005) Analysis of the virus population present in equine faeces indicates the presence of hundreds of uncharacterized virus genomes. Virus Genes 30:151–156

Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D (2007) Experimental phage therapy against Staphylococcus aureus in mice. Antimicrob Agents Chemother 51:2765–2773

Chen CY et al (2003) Comparative genome analysis of Vibrio vulnificus, a marine pathogen. Genome Res 13:2577–2587

Cho JC, Giovannoni SJ (2004) Cultivation and growth characteristics of diverse group of oligotrophic marine gammaproteobacteria. Appl Environ Microbiol 70:432–440

Connon SA, Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl Environ Microbiol 68:3878–3885

Courtois S, Cappellano CM, Ball M, Francou FX, Normand P, Helynck G, Martinez A, Kolvek SJ, Hopke J, Osburne MS, August PR, Nalin R, Guerineau M, Jeannin P, Simonet P, Pernodet JL (2003) Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Appl Environ Microbiol 69:49–55

Crameri R, Suter M (1993) Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for the selection of functional gene products linked to the genetic information responsible for their production. Gene 137:69–75

Daniel R (2005) Metagenomics of soil. Nat Rev Microbiol 3:470–478
Delong EF (2005) Microbial community genomics in the ocean. Nat Rev Microbiol 3(6):459–469
DeSantis G et al (2002) An enzyme library approach to biocatalysis: development of nitrilases for enantioselective production of carboxylic acid derivatives. J Am Chem Soc 124:9024–9025
Detter JC et al (2002) Isothermal strand-displacement amplification applications for high-throughput genomics. Genomics 80:691–698
Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N, Wilson M, Mullany P (2003) Novel tetracycline resistance determinant from the oral metagenome. Antimicrob Agents Chemother 47:1430–1432
Dunbar J, Takala S, Barns SM, Davis JA, Kuske CR (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 65:1662–1669
Dunbar J, Barns SM, Ticknor LO, Kuske CR (2002) Empirical and theoretical bacterial diversity in four Arizona soils. Appl Environ Microbiol 68:3035–3045
Easton S (2009) Functional and metagenomic analysis of the human tongue dorsum using phage display: submitted for the degree of Doctorate of Philosophy. http://discovery.ucl.ac.uk/18512/
Eden PA, Schmidt TM, Blakemore RP, Pace NR (1991) Phylogenetic analysis of Aquaspirillum magnetotacticum using polymerase chain reaction-amplified 16S rRNA-specific DNA. Int J Syst Bacteriol 41:324–325
Falkowski PG, de Vargas C (2004) Shotgun sequencing in the sea: a blast from the past? Science 304:58–60
Frank DN, Zhu W, Sartor RB, Li E (2011) Investigating the biological and clinical significance of human disbioses. Trends Microbiol 19:427–434
Futamata H et al (2001) Group-specific monitoring of phenol hydroxylase genes for a functional assessment of phenol-stimulated trichloroethylene bioremediation. Appl Environ Microbiol 67:4671–4677
Gabor EM, de Vries EJ, Janssen DB (2003) Efficient recovery of environmental DNA for expression cloning by indirect methods. FEMS Microbiol Ecol 44:153–163
Gilbert JA, Field D, Huang Y, Edwards R, Li W et al (2008) Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities. PLoS One 3:e3042
Gill RS et al (2006) Metagenomic analysis of the human distal gut microbiome. Science 312(5778):1355–1359
Gillespie DE, Brady SF, Bettermann AD, Cianciotto NP, Liles MR, Rondon MR, Clardy J, Goodman RM, Handelsman J (2002) Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl Environ Microbiol 68:4301–4306
Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63
Gnanasekar M, Rao KV, He YX, Mishra PK, Nutman TB, Kaliraj P, Ramaswamy K (2004) Novel phage display-based subtractive screening to identify vaccine candidates of Brugia malayi. Infect Immun 72:4707–4715
Green CD et al (2001) Open systems: panoramic views of gene expression. J Immunol Methods 250:67–79
Guan C, Ju J, Bradley BR et al (2007) Signal mimics derived from a metagenomic analysis of the gypsy moth gut microbiota. Appl Environ Microbiol 73(11):3669–3676
Hallam SJ et al (2004) Reverse methanogenesis: testing the hypothesis with environmental genomics. Science 305:1457–1462
Handelsman J (2004) Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68:669–685
Healy FG, Ray RM, Aldrich HC, Wilkie AC, Ingram LO, Shanmugam KT (1995) Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. Appl Microbiol Biotechnol 43:667–674
Henne A, Daniel R, Schmitz RA, Gottschalk G (1999) Construction of environmental DNA libraries in Escherichia coli and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. Appl Environ Microbiol 65:3901–3907
Henne A, Schmitz RA, Bomeke M, Gottschalk G, Daniel R (2000a) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. Appl Environ Microbiol 66: 3113–3116
Henne A, Schmitz RA, Bomeke M, Gottschalk G, Danie R (2000b) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli. Appl Environ Microbiol 66:3113–3116
Hoogenboom HR, De Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC (1998) Antibody phage display technology and its applications. Immunotechnology 4:1–20
Homann MJ et al (2004) Rapid identification of enantioselective ketone reductions using targeted microbial libraries. Tetrahedron 60:789–797
Hughes, DS, Felbeck H, Stein LJ (1997) A histidine protein kinasehomolog from the endosymbiont of the hydrothermal vent tubeworm Riftia pachyptila. Appl Environ Microbiol 63:3494–3498
Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions acidobacteria, actinobacteria, proteobacteria, and verrucomicrobia. Appl Environ Microbiol 68:2391–2396
Kaeberlein T, Lewis K, Epstein SS (2002) Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129
Kawarabayasi Y et al (1999) Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, Aeropyrum pernix K1. DNA Res 6(83–101):145–152
Kruger M et al (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. Nature 426:878–881

Lane J (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA 82:6955–6959

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol WG, Prosser IJ, Schuster CS, Schleper C (2006) Archea predominate among ammonia oxidizing prokaryotes in soils. Environ Microbiol 442:806–809

Lloyd-Jones G, Hunter DWF (2001) Comparison of rapid DNA extraction methods applied to contrasting New Zealand soils. Soil Biol Biochem 33:2053–2059

Lorenz P, Eck J (2005) Metagenomics and industrial applications. Nat Rev Microbiol 3:510–516

Lorenz P, Liebeton K, Niehaus F, Eck J (2002) Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr Opin Biotechnol 13:572–577

MacLean D, Jones GDJ, Studholme JD (2009) Application of 'next generation' sequencing technologies to microbial genetics. Nat Rev Microbiol 7:287–296

Majernik A, Gottschalk G, Daniel R (2001) Screening of environmental DNA libraries for the presence of genes conferring Na+(Li+)/H+antiporter activity on Escherichia coli: characterization of the recovered genes and the corresponding gene products. J Bacteriol 183:6645–6653

Matthews LJ, Davis R, Smith GP (2002) Immunogenically fit subunit vaccine components via epitope discovery from natural peptide libraries. J Immunol 169:837–846

McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348:552–554

Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Gorski A (2007) Phage therapy of staphylococcal infections (including MRSA) may be less expensive than antibiotic treatment. Postepy Hig Med Dosw (Online) 61:461–465

Miller DN et al (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Appl Environ Microbiol 65:4715–4724

Mitra S, Schubach M, Hudson HD (2010) Short clones or long clones? A simulation study on the use of paired reads in metagenomics. BMC Bioinformatics 11(Suppl 1):S12.1–S12.11

Morris RM, Rappe MS, Connon SA, Vergin KL, Siebold WA, Carlson CA, Giovannoni SJ (2002) SAR11 clade dominates ocean surface bacterioplankton communities. Nature 420:806–810

Mullen LM, Nair SP, Ward JM, Rycroft AN, Henderson B (2006) Phage display in the study of infectious diseases. Trends Microbiol 14:141–147

Nelson KE et al (1999) Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of Thermotoga maritima. Nature 399:323–329

Ochman H, Jose Ayala F, Hartl DL (1993) Use of polymerase chain reaction to amplify segments outside boundaries of known sequences. Methods Enzymol 218:309–332

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. J Microbiol Methods 7:57–66

Ovreas L (2000) Population and community level approaches for analysing microbial diversity in natural environments. Ecol Lett 3:236–251

Pace NR, Stahl DA, Lane DJ, Olsen GJ (1986) The analysis of natural microbial populations by ribosomal RNA sequences. Adv Microb Ecol 9:1–55

Pedulla ML et al (2003) Origins of highly mosaic mycobacteriophage genomes. Cell 113:171–182

Piel J (2004) Metabolites from symbiotic bacteria. Nat Prod Rep 21:519–538

Preston CM, Wu KY, Molinski TF, DeLong EF (1996) A psychrophilic crenarchaeon inhabits a marine sponge: Cenarchaeum symbiosum gen. nov., sp. nov. Proc Natl Acad Sci USA 93:6241–6246

Prinz DM, Smithson SL, Westerink MA (2004) Two different methods result in the selection of peptides that induce a protective antibody response to Neisseria meningitidis serogroup CJ. Immunol Methods 285:1–14

Qin J et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65. doi:10.1038

Renesto P et al (2003) Genome-based design of a cell-free culture medium for Tropheryma whipplei. Lancet 362:447–449

Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6:981–989

Robertson DE et al (2004) Exploring nitrilase sequence space for enantioselective catalysis. Appl Environ Microbiol 70:2429–2436

Robinson JJ, Stein JL, Cavanaugh CM (1998) Cloning and sequencing of a form II ribulose-1,5-biphosphate carboxylase/oxygenasefrom the bacterial symbiont of the hydrothermal vent tubeworm Riftia pachyptila. J Bacteriol 180:1596–1599

Rondon MR et al (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Schloss PD, Handelsman J (2003) Biotechnological prospects from metagenomics. Curr Opin Biotechnol 14:303–310

Simon C, Daniel R (2011) Metagenomic analyses: past and future trends. Appl Environ Microbiol 77:1153–1161

Smith GP, Petrenko VA (1997) Phage display. Chem Rev 97:391–410

Stahl DA, Lane DJ, Olsen GJ, Pace NR (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl Environ Microbiol 49:1379–1384

Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF (1996) Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment front a planktonic marine archaeon. J Bacteriol 178:591–599

Tamas I et al (2002) 50 million years of genomic stasis in endosymbiotic bacteria. Science 296:2376–2379

Teeling H, Meyerdierks A, Bauer M, Amann R, Glockner FO (2004) Application of tetranucleotide frequencies for the assignment of genomic fragments. Environ Microbiol 6:938–947

Trepel M, Arap W, Pasqualini R (2002) In vivo phage display and vascular heterogeneity: implications for targeted medicine. Curr Opin Chem Biol 6:399–404

Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS, Banfield JF (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43

Uchiyama T, Abe T, Ikemura T, Wantanabe K (2005) Substrate-induced gene expression screening of environmental metagenome libraries for isolation of catabolicgenes. Nat Biotechnol 23:88–93

Uchiyama T, Miyazaki K (2010) Product-induced gene expression (PIGEX): a product-responsive reporter assay for enzyme screening of metagenomic libraries. Appl Environ Microbiol 76(21):7029–7035

Urbach E et al (1999) Immunochemical detection and isolation of DNA from metabolically active bacteria. Appl Environ Microbiol 65:1207–1213

Vakhlu J, Sundan KA, Johri NB (2008) Metagenomics: Future of microbial gene mining. Indian J Microbiol 48:202–215

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74

Vezzi A et al (2005) Life at depth: Photobacterium profundum genome sequence and expression analysis. Science 307:1459–1461

Wagenaar JA, Van Bergen MA, Mueller MA, Wassenaar TM, Carlton RM (2005) Phage therapy reduces Campylobacter jejuni colonization in broilers. Vet Microbiol 109:275–283

Warnecke F, Hess M (2009) A perspective: metatranscriptomics as a tool for the discovery of novel biocatalysts. J Biotechnol 142:91–95

Weber-Dabrowska B, Mulczyk M, Gorski A (2000) Bacteriophage therapy of bacterial infections: an update of our institute's experience. Arch Immunol Ther Exp (Warsz) 48:547–551

Wilkinson DE et al (2002) Efficient molecular cloning of environmental DNA from geothermal sediments. Biotechnol Lett 24:155–161

Williamson LL, Bradley RB, Schloss PD et al (2005) Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. Appl Environ Microbiol 71(10):6335–6344

Wilmes P, Bond LP (2004) The application of two dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environ Microbiol 6:911–920

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Wommack KE, Ravel J, Hill RT, Chun J, Colwell RR (1999) Population dynamics of Chesapeake Bay virioplankton: total-community analysis by pulsed field gel electrophoresis. Appl Environ Microbiol 65:231–240

Yeager CM et al (2004) Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert. Appl Environ Microbiol 70:973–983

Yun J, Ryu S (2005) Screening for novel enzymes from metagenome and SIGEX as a way to improve it. Microb Cell Fact 4:1–5

Zengler K et al (2002) Cultivating the uncultured. Proc Natl Acad Sci USA 99:15681–15686

Zhou J et al (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl Environ Microbiol 68:326–334

Genetically Modified Microorganisms (GMOs) for Bioremediation

11

Sandeep Kumar, Vikas Kumar Dagar, Yogender Pal Khasa, and Ramesh Chander Kuhad

Abstract

The increasing amount of pollutants in the environment is an alarming concern to the ecosystem. A number of organic pollutants, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and pesticides, are resistant to degradation, which represent toxological threat to wildlife as well as human beings. Various physiological and biological measures have been employed globally to degrade these hydrocarbons to improve environment quality. Out of these, bioremediation is the most promising strategy where microorganisms are harnessed to degrade the organic and inorganic pollutants. There are many naturally existing microbes, which are routinely employed in bioremediation process. At instances, these consortia of microorganisms in various environmental conditions provide an insight about the interrelation of metabolic pathways involved in biodegradation process. Various metabolic techniques are employed to produce genetically engineered microorganisms (GEMs) with better bioremediation efficiency. Majorly biomolecular engineering approaches such as rational designing and directed evolution have been developed to genetically modify microorganisms and their enzymes for the degradation of persistent organic pollutants (POPs) like PAHs, PCBs, and pesticides. Recently, several developments in the field of recombinant DNA technologies such as development of "suicidal-GEMs" (S-GEMs) have also been carried out to achieve safe and efficient bioremediation of contaminated sites. In this chapter, we describe various techniques for the development of genetically modified microorganisms along with different examples of recombinant produced. Harmful impact of the engineered microorganisms on environment and economic consideration of viable processes development are critically discussed.

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Bioremediation • Genetically engineered microorganisms (GEMs) • Rational designing • Directed evolution • Metabolic engineering • Suicidal-GEMs

Introduction

Accumulated toxic and persistent compounds especially xenobiotics in the environment cannot be degraded efficiently by the natural metabolic diversity of the autochthonous microbes. The novel nature of these pollutants which includes unusual chemical bonding or substitutions with halogens or other functional groups makes them resistant to natural degradation by the inhabiting microorganisms (MOs), due to the lack of appropriate metabolic pathway. Hydrophobic nature of various toxic contaminants in environment such as toluene, organochlorides, and polyaromatic hydrocarbons accumulates in the microbial cell and disrupts their cell membrane (Pieper and Reineke 2000). The prolonged persistence of these compounds is due to the inefficient transport systems and their limited bioavailability because of their hydrophobic nature. However, the natural inhabiting consortia at various physiological environmental conditions provide the genetic sources of novel metabolic capabilities (Pieper and Reineke 2000) and the possibilities of degrading these recalcitrant chemicals using genetically modified bacterial strains through the introduction of different enzyme activities from a number of bacteria into a desired microorganism (Chen and Mulchandani 1998). Microorganisms have been evolving since last 3.8 billion years and inhabit virtually in all environmental conditions like extremes of nutrient concentration, salinity, pH, pressure, and temperature (DeLong and Pace 2001). During the long evolutionary process, different microorganisms gained unique abilities to degrade persistent compounds at contaminated sites by the physiochemical pressure in those environmental conditions. The potential of this natural microflora can be utilized by identifying and engineering catabolic gene pool. The use of indigenous microflora instead of exotic strains is always naturally advantageous for the construction of recombinant microorganisms because the indigenous microorganisms are more prone to interact with total population and can withstand to the complex stressful environmental conditions (Singh et al. 2011). Catechol 1,2-dioxygenase, toluene dioxygenase-iron-sulfur protein component, benzene dioxygenase, naphthalene dioxygenase (Moharikar et al. 2003), dioxin dioxygenase of *Sphingomonas* sp. strain RW1 (Armengaud et al. 1998), carbazole-1,9-dioxygenase (Sato et al. 1997), and many ring-activating dioxygenases (Fuenmayor et al. 1998) are some of the examples of novel enzymes, identified for recombinant DNA technology to improve strains for better bioremediation capabilities.

A multidisciplinary involvement of microbiology, molecular biology, biochemistry, bioinformatics, and genomics is required to produce genetically engineered microorganisms (GEMs) to overcome various bottlenecks in the cleanup of contaminated sites. There are very few reports where GEMs have been applied and proven to be more efficient than natural MOs in elimination of recalcitrant compounds under natural conditions (de Lorenzo 2009). However, efforts are made to expand the range of compounds that can be degraded by applying the principles of recombinant DNA technology (Keasling and Bang 1998; Timmis and Pieper 1999; Ryu and Nam 2000; Ang et al. 2005; Liu et al. 2006; Kapley and Purohit 2009). For bioremediation purpose, the first test release of genetically engineered microorganisms was commenced by the US Environmental Protection Agency (EPA) in 1996 (Ryan et al. 2000). The successful application of genetically MOs for bioremediation is based upon the successful establishment of the engineered microorganisms in the environmental conditions and after the completion of the desired

objectives; there should be an appropriate mechanism for their removal from the site of action (Carlos and Alkorta 1999). The potential risks associate with the release of GEMs into the environment has originated the necessity to construct the biological containment systems by which bacteria are killed in a controlled suicide process (Carlos and Alkorta 1999). The mechanisms designed by various research groups to develop GMOs are discussed in detail in this chapter.

Recombinant DNA Technologies Used for Microorganism Modifications

The strategies used for engineering any microbial cell capable of degrading specific compounds and also to make it able to remain viable for many generations are very different from the techniques used to develop recombinant strains for protein overproduction. A balance between the organism growth and energy consumption for the macromolecule synthesis, capability of the genetic vector to carry the large foreign DNA, ability of the cell to replicate the DNA exactly, and maintenance of the plasmid by the cell are some of the issues which must be kept in mind during designing any strategy (Keasling and Bang 1998). To develop an efficient microorganism, the number of expression systems containing various promoters and their regulators has been discovered by different groups (de Lorenzo et al. 1993; Endo and Silver 1995). Carrier and Keasling (1997) designed a technique to enhance the stability of mRNA by introduction of DNA cassettes at 5′ untranslated region of a desired gene. The incorporated DNA cassette forms hairpin at the 5′ end of the mRNA and increased the ΔG of formation of that mRNA, which in turn increased mRNA stability by threefolds. As far as cloning of the genes responsible for recalcitrant compounds degradation is concerned, the broad-host-range cloning vectors such as RK2 (Kolatka et al. 2010), TFK (Kalyaeva et al. 2002), and pPP8 (Holtwick et al. 2001) and the narrow-host-range plasmids cloning vectors such as pDK1, pFME4, pFME5, pNK33 (Izmalkova et al. 2005; Shintani et al. 2005), and pPS10 (Nieto et al. 1990) for *Pseudomonas* and other bacteria have been designed.

The modification of enzyme affinity and specificity, bioprocess development, pathway construction and regulation, bioactivity bioreporter sensor development for chemical sensing, end-point analysis, and toxicity reduction are the main approaches to develop GEMs. The commonly used strategies to develop recombinant microorganisms for bioremediation applications (Fig. 11.1) have been discussed in detail in the following sections.

Rational Designing

The rational designing involves the construction of a single microorganism having the assembly of desirable biodegradation pathways or enzymes from different organisms to perform specific reactions using recombinant DNA technology (Ang et al. 2005). Prior information on the mechanistic, dynamic, and structural properties of protein is required to successfully modify it by rational design for the specific purposes as delicate folding kinetics of any protein is highly prone to disrupt its confirmation leading loss of catalytic activity. Nonetheless, there is rapid improvement in the capabilities of rational design techniques due to the involvement of more efficient X-ray crystallography and functional bioinformatics techniques. There are many reports which are concerned with the improvement in the properties of different enzymes and other proteins. Kellner et al. (1997) modified P450 monooxygenase systems in order to improve its substrate specificity and catalytic efficiency. The knowledge of the structural and functional relationships in various cytochrome P45Os provided the basis for the rational design of P45Os to catalyze desired biodegradation reactions. It was found that modulations in the substrate binding pocket even by single amino acid provided enhancement in the flexibility of the binding affinity of this enzyme. In another report, nitric oxide reductase (NOR), which is the key enzyme of nitrogen cycle that is critical for all life forms,

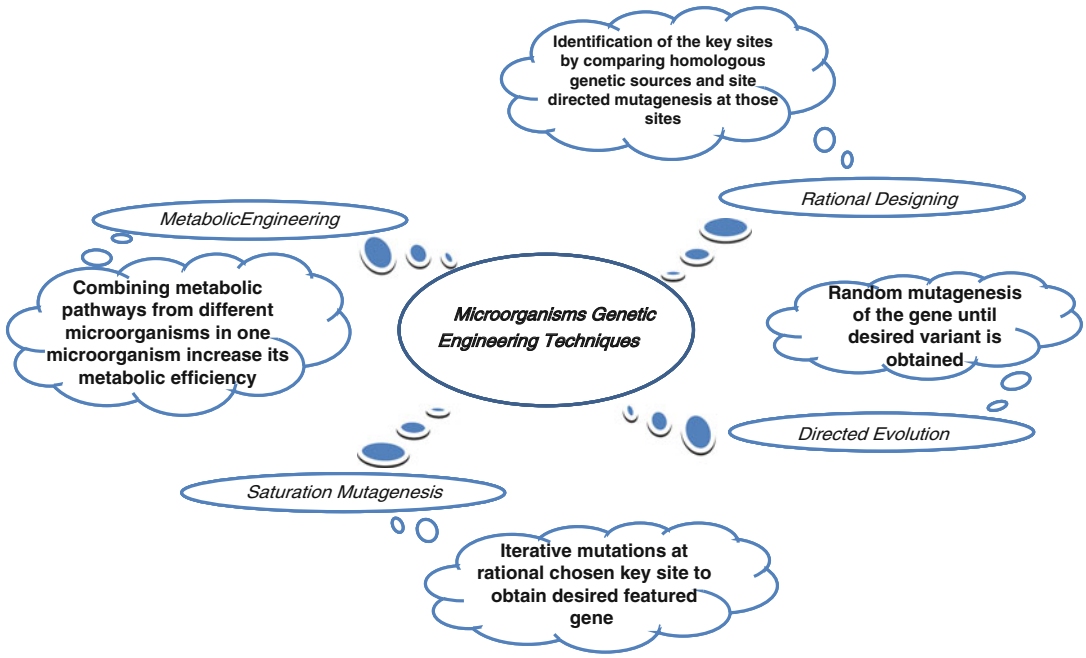

Fig. 11.1 Different approaches to develop genetically modified microorganisms for bioremediation

was rationally modified by the introduction of three histidine and one glutamate, predicted as ligands in the active site of NOR, into the distal pocket of myoglobin (Natasha et al. 2009). Similarly, haloalkane dehalogenase enzyme which hydrolyzes halogenated aliphatics was engineered for mutational analysis by studying the kinetics of the reaction catalyzed after replacing Phe172 by tryptophan. Phe172 is located in helix-loop-helix structure that covers the active site cavity of the enzyme, and it is believed to stabilize the structure of the enzyme. The engineered enzyme showed tenfold higher Kcat/Km than the wild type (Schanstra et al. 1996).

An encouraging example of the rationally designed catabolic pathway was represented by Mattozzi et al. A strain of *Pseudomonas putida* was constructed to degrade an organophosphorus compound paraoxon which was capable to utilize this compound as a sole carbon and nitrogen source after the mutations. The *pnp* operon from *Pseudomonas* sp. strain ENV2030 encoding enzymes that transform p-nitrophenol into β-ketoadipate was inserted into the organism with a simultaneous expression of a synthetic operon encoding a phosphodiesterase (encoded by *pde*) from *Delftia acidovorans*, an organophosphate hydrolase (encoded by *opd*) from *Flavobacterium* sp. strain ATCC 27551, and an alkaline phosphatase (encoded by *phoA*) from *Pseudomonas aeruginosa* HN854 (Mattozzi et al. 2006). This whole assembly was programmed to be regulated by a constitutive promoter. The recombinant strain was able to utilize about 275 g/l of paraoxon as energy source (Mattozzi et al. 2006). The general mechanism of modifying a microorganism or an enzyme by rational modification is depicted in Fig. 11.2.

Directed Evolution

Darwinian principles of mutation and selection are the basis for the improvement of biocatalysis and cellular properties by *directed evolution* (Chatterjee and Yuan 2006). This technology involves the engineering of the enzymes with desired characteristics using site-directed mutagenesis

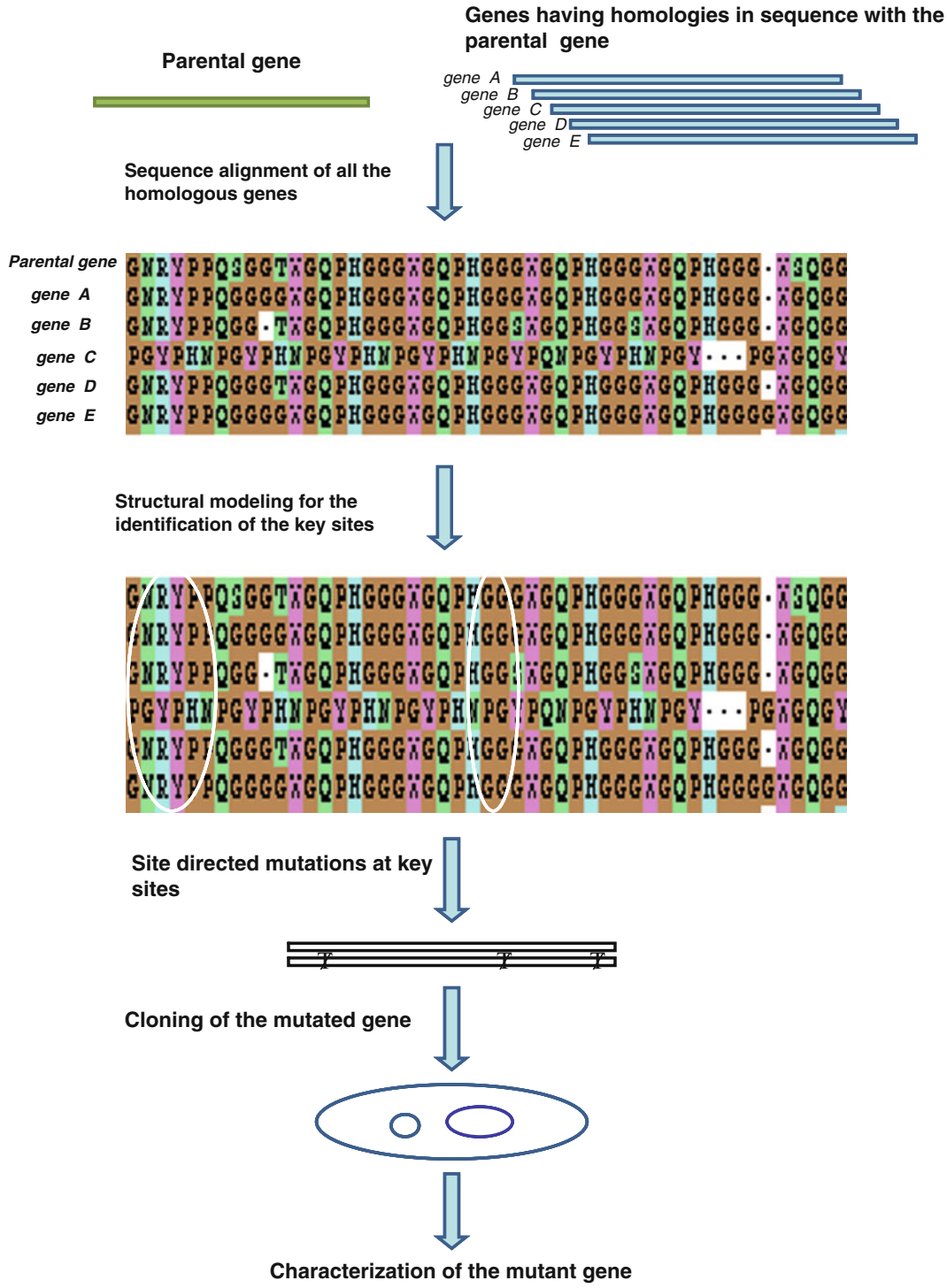

Fig. 11.2 Schematic representation for the development of genetically modified microorganism using rational design

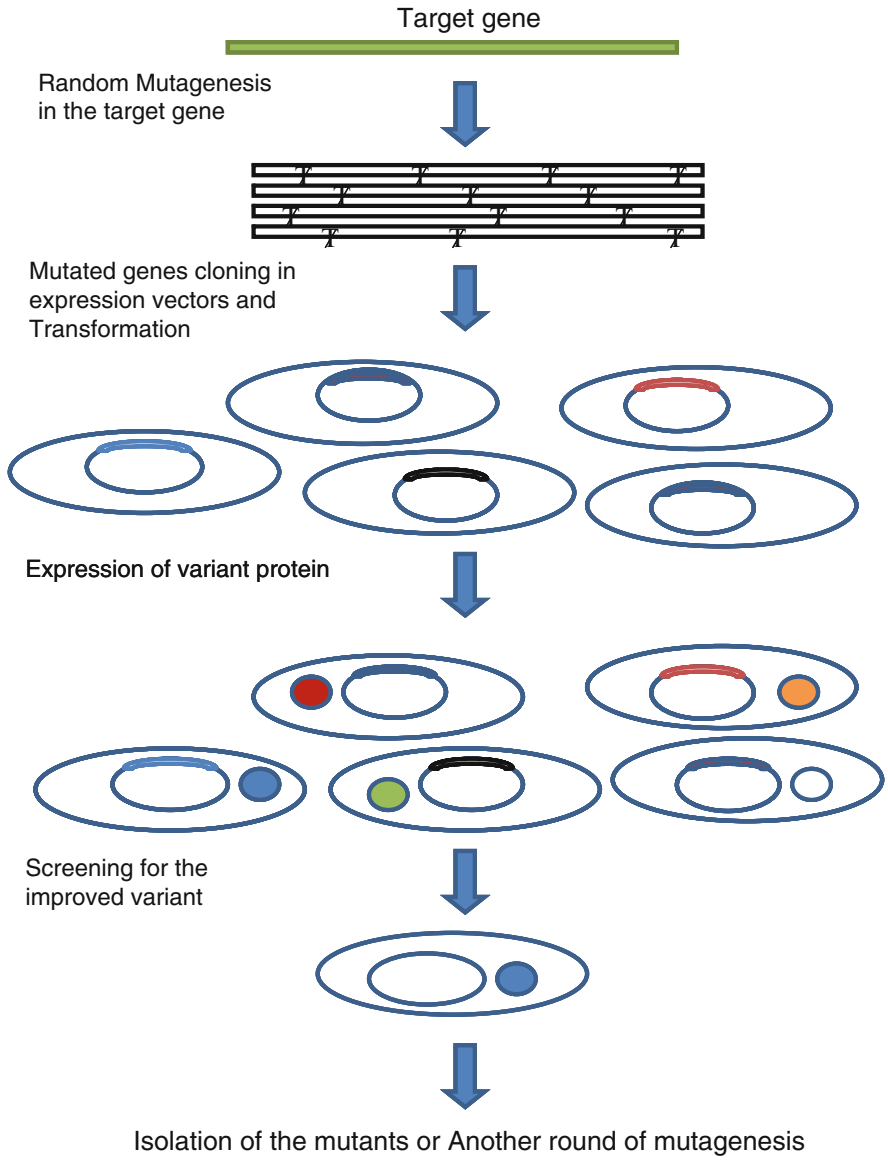

Fig. 11.3 Schematic representation for development of genetic variants using directed evolution approach

(Fig. 11.3). In vitro directed evolution mimics natural processes like random mutagenesis and sexual recombination, and it does not require detailed understanding of the structure and properties of the target protein (Hibbert and Dalby 2005). This strategy uses PCR methodology without primers to reassemble a gene from DNA fragments of variable lengths of about 10–300 bps generated by predigestion of the gene with nucleases (Stemmer 1994). After assembly of different size fragments by homologous recombinations and further extensions with polymerase using dNTPs, a nested PCR strategy is applied to yield full length mutated gene (Wood 2008).

Despite various technical advancements in the field, there are still some constraints such as difficulty in optimizing ligation steps for making

large size libraries and library screening (Hibbert et al. 2005) which makes the process of directed evolution impractical in many cases. Adopting PCR-based approaches and directing in vivo hyper-mutation with B cells to target genes delivered by retroviral infection are the improvements to circumvent the ligation-related issues (Wang et al. 2004). Improvement in the proportion of nonredundant or degenerate variants in libraries could be a solution for practical limitation to library size (Hamamatsu et al. 2005). A more recent finding has shown that the majority of mutations for the enhancement in the enantioselectivity of enzymes occur within 10 A° of the enzyme active site (Hibbert and Dalby 2005). With incorporation of such observations, focused mutagenesis approaches are being used to obtain more efficient enzymes (Reetz et al. 2005; Parikh and Matsumura 2005: Morley and Kazlauskas 2005). Further, enhancement in the property of enzyme has been achieved by using consensus sequence data for library construction (Amin et al. 2004). By aligning the target gene with the consensus sequence from the homologues, some potential residues had been identified. Furthermore, these sites were mutated to produce a combinatorial library with a good success profile.

Saturation Mutagenesis

Engineered catalysts having mutations at key sites are developed using this technique. Key sites are targeted for iterative cycles of mutagenesis until the gene for the desired level of enhancement in the gene property is achieved. The choice of target site depends upon the property which needs to modify to obtain better characteristics (Reetz and Carballeira 2007). Focused libraries are generated by random changes in amino acids sequence where changes can be incorporated at one position or simultaneously at different positions. The main advantage of saturation mutagenesis or cassette mutagenesis is that the libraries produced using this technique are easy to screen because the modified sequence space is small (Reetz and Carballeira 2007; Ang et al. 2007, 2009). For example, enhancement in the activity of aniline dioxygenase (AtdA) isolated from *Acinetobacter* sp. strain YAA was achieved in order to degrade aromatic amines more efficiently. Modification in the activity was obtained by one round of saturation mutagenesis followed by three rounds of random mutagenesis. The activity of the final mutant was found to be enhanced by 8.9-, 98.0-, and 2.0-fold for aniline, 2,4-dimethylaniline (2,4-DMA), and 2-isopropylaniline (2-IPA), respectively (Ang et al. 2009). Significant increase in the activity towards m-nitrophenol (34 times), o-nitrophenol (47 times), and o-methoxyphenol (174 times) as well as an expanded substrate range in comparison to wild-type organism was achieved by producing V350F 2,4-dinitrotoluene dioxygenase mutant of *Burkholderia cepacia* R34 by saturation mutagenesis (Keenan et al. 2004). Similarly, another mutant V350M of the same enzyme displays enhanced activity towards o-nitrophenol (20 times) and o-methoxyphenol (162 times) as well as novel activity towards o-cresol (Keenan et al. 2004). Saturation mutagenesis relies on the detailed analysis of the region of the protein crucial for the desired catalytic property. In this prospect, it constitutes a symbiosis between rational design and combinatorial randomization. The general scheme of saturation mutagenesis is detailed as shown in Fig. 11.4.

A combination of DNA shuffling and saturation mutagenesis has been used to engineer the gene for catalyst toluene-o-xylene monooxygenase from *P. stutzeri* OX1 to enhance the degradation of chlorinated ethane and also to discover new residues for accelerating *p*-nitrophenol degradation (Vardar and Wood 2005; Radice et al. 2006). Similarly, nitrophenolic pollutants such as dinitrotoluenes, 4-methyl-5-nitrocatechol, and aminonitrotoluenes were degraded by evolving monooxygenases and dioxygenases (Leungsakul et al. 2006). Bacterial systems have also been created which can utilize 1,2,3-trichloropropane (TCP) as a source of carbon and energy. Haloalkane dehalogenase from *Rhodococcus* sp. M15-3 has been evolved to allow more productive binding of TCP in the active site, and the gene for this enzyme was expressed in *Agrobacterium*

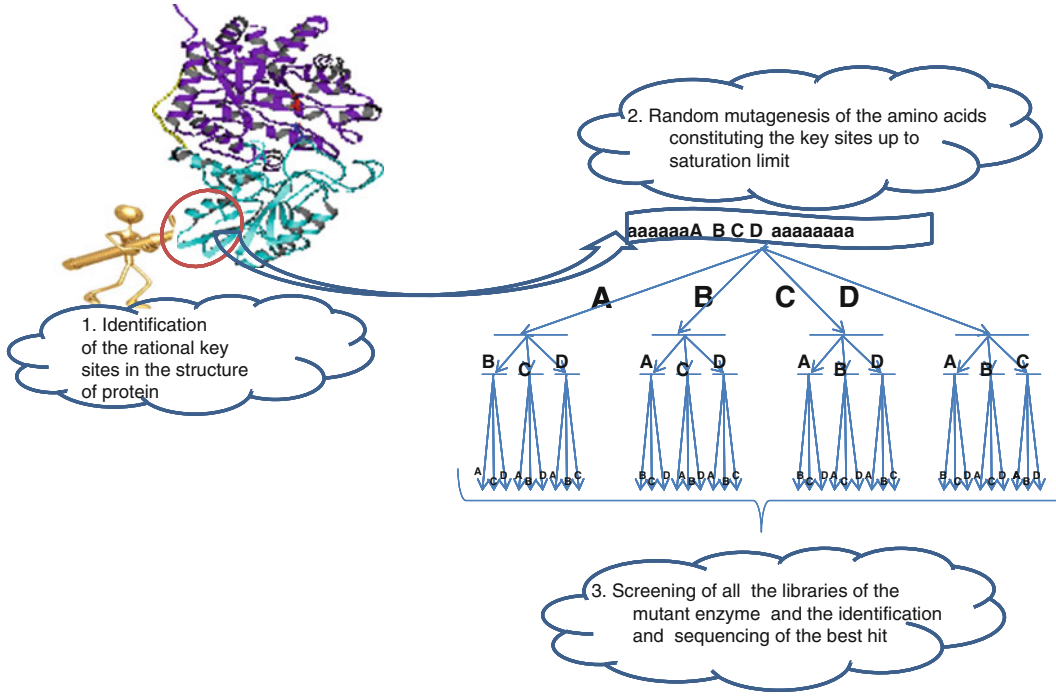

Fig. 11.4 Saturation mutagenesis and GEM development

radiobacter AD1, which helped in fast degradation of TCA at contaminated sites (Bosma et al. 2002).

Protein engineering by *genomic shuffling* is the process of recombination of the chromosomes from several bacteria to produce a bacterium which has the improved activity of a desired trait. The degradation of the pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723 has been improved by genomic shuffling up to tenfold in comparison to wild-type strain (Zhang et al. 2002; Dai and Copley 2004). In contrast to genome shuffling, *family shuffling* involves the shuffling of the DNA of the related groups of the genes to accelerate the directed evolution. The evolution of the hybrid enzymes with enhanced polychlorobiphenyls (PCBs) degradation potential by the family shuffling of the genes for the large subunit of biphenyl dioxygenase (*bphA*) from *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 is one of the pioneer applications of protein engineering for bioremediation (Kumamaru et al. 1998).

Metabolic Engineering

The process of the enhancement in the production of a specific cellular compound by the optimization of genetic and regulatory means is regarded as metabolic engineering. In this industrialized world, the ultimate goal of engineering any pathway in a microorganism is to change the properties of a cell in order to achieve desirable cellular traits for bio-processing. However, if the metabolic engineering for the purpose of the degradation of harmful pollutants is considered, then one of the ways could be the enhancement in the metabolic capability of a microorganism by combining the metabolic pathways from different organisms in a single bacterium. One of the finest examples of metabolic engineering was the degradation of chloro- and methylaromatics, where five different catabolic pathways from three different bacteria were made to combine in order to degrade methylphenols and methylbenzoates in a bacterium (Rojo et al. 1987).

Oxygenases are having significant impact on the degradation of various xenobiotic pollutants present in the soil. Nowadays, these enzymes are being considered as potential target enzymes for metabolic engineering. This class of biocatalysts simply incorporates oxygen from the environmental O_2 in the structure of the organic compounds in order to oxidize them (Fetzner 2007). Dioxygenase of the toluene dioxygenase system of *Pseudomonas putida* F1 which transforms 2-chlorotoluene into 2-chlorobenzaldehyde by virtue of its residual methyl-monooxygenase activity on o-substituted substrates, benzyl alcohol dehydrogenase (encoded by xylB), and benzaldehyde dehydrogenase (xylC) present in pWWO plasmid of *P. putida* mt-2 was assembled in separate mini-Tn5 transposon vectors. This gene cassette was inserted in the chromosome of the 2-chlorobenzoate degraders *Pseudomonas aeruginosa* PA142 and *P. aeruginosa* JB and expressed under toluene-responsive Pu promoter of the TOL plasmid and the cognate XylR regulator (Haro and deLorenzo 2001). Although the mineralization efficiency of this assembly was not found to be as expected, but the resultant strain possessed the genetic information and functional capabilities to mineralize the target pollutant (Haro and deLorenzo 2001). Though, further optimization strategies need to be done.

The degradation of *cis*-1,2-dichloroethylene (*cis*-DCE) has been enhanced by metabolically engineered *E. coli* strain. The modified strain expressed six genes of an evolved toluene orthomonooxygenase (TOM) from *Burkholderia cepacia* G4 with either gamma-glutamylcysteine synthetase (GSHI) or the glutathione S-transferase IsoILR1 from *Rhodococcus* AD45 or with an evolved epoxide hydrolase from *Agrobacterium radiobacter* AD1 which converts the reactive *cis*-DCE epoxide to a diol. The impact of this metabolic engineering on bioremediation was accessed by observing the changes in the proteome through a quantitative shotgun proteomics technique (iTRAQ) by the sequential addition of TOM-Green, IsoILR1, and GSHI when the comparison was made with the wild-type strain. This technique leads to increased aerobic degradation of *cis*-DCE and reduced toxicity from *cis*-DCE epoxide (Lee et al. 2006).

Whole-transcriptome profiling is another molecular technique that has also been employed by various research groups to determine mutualistic interactions in the rhizosphere for strains relevant for bioremediation. Some primitive studies in this regard have been done in order to understand the metabolism of bacteria in the rhizosphere (Okinaka et al. 2002; Attila et al. 2008). As an example, *Shewanella oneidensis,* which is an important model organism for bioremediation due to its diverse respiratory capability, has been subjected to whole genome profiling by microarray analysis. The purpose of this analysis was to find the molecular basis for the change in the expression level of the temporal genes when the cells were subjected to heat stress. It was found that majority of the genes which were homologous to the chaperones and heat shock proteins in other organisms were highly induced (Gao et al. 2004).

Even after considering all the above techniques, the problem still persists with regard to programming any microorganism physiologically and genetically to express the desired trait at expected level and at the right time under physiological circumstances. Although plasmids are playing lead role in the production of recombinant microorganisms, but they are having their own limitations such as their instability in the absence of selective pressure and their potential for lateral transfer of recombinant genes to indigenous bacteria (de Lorenzo 1994). These difficulties with plasmids have resulted in the enhanced use of mini-transposons as engineering tools where it allows very stable recombinant phenotypes to be engineered with very less number of manipulations (de Lorenzo 1994).

Although the transposon system is generally used as a mean to upgrade the feasibility of the expression of a gene in heterologous environment, even then, sometimes there come the issues like the different behavior of translation initiation regions (TIRs). These issues are covered by the use translational enhancers, which are 40–50 bp sequences and generally substitute functionality of Shine-Dalgarno (SD) sequence. These sequences can enhance the translation of a particular sequence in a wide variety of hosts. As an example, the

expression of chloramphenicol acetyl transferase (CAT) in various microbes has been enabled by a translational enhancer from tobacco mosaic virus, when it was placed at different distances upstream of the initiator AUG codon (Gallie and Kado 1989).

A bit apart from all these above strategies, the use of regulated promoters of the biodegradative pathways is very attractive for designing heterologous expression system in the field. The *Pm, Pu*, and *Psal* are some of the best characterized promoters of catabolic plasmids TOL and NAH of *Pseudomonas* sp. (Burlage et al. 1989). The specific properties of these promoters are that they can be induced cost effectively; they all have a broad range and can function in a number of genera (Schweizer 2001). For example, 1 ppm of benzoate can activate the *Pm* promoter of the TOL plasmid pWW0 of *P. putida* at reasonable levels (de Lorenzo 1994).

The mRNA instability is also an obstacle in the heterologous expression of a sequence. Bacteriophage T4 gene 32 exhibits an interesting property related to structure and processing of its leader untranslated 5′ end which is responsible for the increased stability of its mRNA (Anderson and Dunman 2009). So the hybrid constructed by composing the native promoter/TIR region of genes 32 and *xylE* (which encodes catechol 2,3-dioxygenase) was expressed at high levels in gram-negative bacteria.

Recombinant Bacteria

The combination of ecological and microbiological knowledge with biochemical mechanisms and field engineering design is essential for successful in situ bioremediation. Besides the ethical issues, there are more factors hampering a successful designing of recombinant strain. Competition of the engineered microorganism with the other natural inhabitants for nutrients and other resources and selection pressure offered by the biotic and abiotic factors are two significant hurdles (Singh et al. 2011). Therefore, choosing a right bacterial strain with respect to growth potential and nutrient response is must to achieve desirable results.

There are numerous reports for the development of recombinant bacterial systems for the degradation of various pollutants. The first genetically engineered microbe was created by Chakrabarty group in 1971. The patent was approved in 1980 by the United States Supreme Court. The microbe was a variant of the genus *Pseudomonas* and was capable of degrading crude oil constituents (Ezezika and Singer 2010).

Biodegradation of Heavy Metals

Heavy metals such as mercury (Hg), arsenic (As), cadmium (Cd), nickel (Ni), cobalt (Co), and lead (Pb) accumulated in the environment due to rapid industrialization are known to cause severe damage to the vital organs in living organisms (Singh et al. 2011). Detoxification machinery of genetic engineered bacteria has been considered useful for metal bioremediation. In response to high Hg toxicity, bacterial world has evolved surprising array of resistance mechanisms. A cluster of Hg resistance genes (*mer*) in an operon encourage the bacteria to detoxify Hg^{2+} into volatile Hg by Hg reductase (Barkay et al. 2003). The narrow Hg resistance *mer* operon has been found to degrade mercury in a series of three consecutive steps, that is, transportation of Hg^{2+} into the cell, enzymatic NADPH-dependent conversion of the ionic mercury into less toxic elementary mercury (Hg^0), and finally the regulation of the functional genes for the transport and transformation (Singh et al. 2011). This operon consists of various genes which are functionally differentiated such as *merA* and *merB* for reduction, *merT* and *merP* as transporter, *mer R* and *merD* for regulation, and recently discovered *merE* and *merH* also for membrane transport (Ruiz and Daniell 2009; Kiyono et al. 2009; Schue et al. 2009). Genetically engineered *E. coli* harboring simultaneously *merT-merP* and MT genes is found to be capable to remove Hg^{2+} effectively from electrolytic waste water (Deng and Wilson 2001). *Deinococcus radiodurans,* the most radiation-resistant bacterium known, was modulated by expressing *merA* gene from *E. coli* BL308. The recombinant produced was found to grow in the presence of radiations as

well as high concentration of ionic mercury. It was able to reduce Hg ions to less toxic volatile elemental mercury efficiently (Brim et al. 2006).

Phytochelatins and metallothioneins, which are metal-binding peptides, have been reported to contribute in the enhancement of heavy metal-binding capabilities of microorganisms (Bae et al. 2001). Genes for these proteins have been cloned in *E. coli* from plants and fungi. Recombinant bacteria with high bioaccumulation capacity and high affinity for target metal selectively accumulate the metal ions from multicomponent pollutants (Sauge-Merle et al. 2003). Use of genetically engineered *E. coli* JM109 for bioaccumulation of Cd^{2+} from a heavy metals polluted site was developed by Deng et al. (2007). Similarly, there are reports on the accumulation of Cd^{2+} by various genetically engineered bacteria such as *Mesorhizobium huakuii, Pseudomonas putida,* and *Caulobacter crescentus* by the expression of fusion proteins for phytochelatin (PC), metal-binding peptide (EC20), and RsaA-6His, respectively (Sriprang et al. 2003; Wu et al. 2010; Patel et al. 2010).

Arsenic (As) possesses high animal health risks because of its affinity to accumulate in the plants edible parts, soil, and contaminated water. Through these sources, this inorganic compound finds its way to the food chain. Keeping in mind the urgent need to remove As from the contaminated site, arsenite S-adenosylmethionine methyltransferase gene (*arsM*) which is taken from *Rhodopseudomonas palustris* has been cloned in *E. coli* (Qin et al. 2006). This enzyme methylated inorganic As to less toxic and volatile trimethylarsine (TMA). Volatilization of As is found to be an efficient way for its removal from contaminated soil by recombinant bacteria which are modulated by *arsM* gene (Liu et al. 2011). The recombinant bacteria were capable to bioaccumulate As at very high concentration compared to control strain. Expression of another metalloregulatory protein ArsR in *E. coli* also promises high affinity to remove As from the contaminated sites (Kostal et al. 2004).

In many organisms, the metallothionein protein (MT), glutathione S-transferase fusion protein (GST-MT), and *nixA*-encoded membrane transport protein exhibited high affinity for membrane transport of Ni^{2+}(Singh et al. 2011). It is presumed that overexpression of these transport proteins would accumulate large amount of Ni^{2+} in the modified bacteria.

Recombinant Microorganisms for Oil and Organic Compounds Degradation

First breakthrough in the biodegradation of oil was observed with the involvement of four strains of *Pseudomonas.* The genes for the degrading enzymes were located on the extrachromosomal part, that is, on the plasmid. The plasmids were isolated and put into a single strain of *Pseudomonas.* These strain showed a capability to degrade oil by 10–100 times faster than the wild type (Time Magazine 1975). *Deinococcus radiodurans*, which is the most radiation-resistant organism known, was genetically engineered for toluene degradation. However, it has not been applied for commercialized bioremediation purposes due to anticipated risks and regulatory controls associated with it (Ezezika and Singer 2010).

Agent Orange is a toxic defoliant used by the United States military during the Vietnam War. This compound is 1*:*1 mixture of two phenoxy herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and it is linked to increased incidences of cancer (Ezezika and Singer 2010). In order to degrade this pollutant, an engineered microbe was produced from a strain of *Burkholderia cepacia*, and it was first tested for the removal of Agent Orange at the US Air Force site in Pensacola, northwestern Florida, where it was stored prior to its shipment to Vietnam (Marwick 2003; Chauhan et al. 2008).

Organophosphate- and carbamate-degrading recombinant strain of *Sphingomonas* sp. CDS-1 was developed by Liu et al. The methyl parathion hydrolase encoding gene (*mpd*) was cloned with cognate regulator of a methyl parathion (MP)-degrading strain *Pseudomonas putida* DLL-1 using shotgun method. Broad-host vector pBBR1MCS-2 was used to produce pBBR-*mpd*

recombinant plasmid which was transformed in *Sphingomonas* sp. CDS-1 to finally produce CDS-pBBR-*mpd* recombinant. The methyl parathione-degrading capacity of the recombinant organism was about 7 times higher than the wild-type strain (Liu et al. 2006).

Degradation of Persistent Organic Compounds Using Recombinant Bacteria

Laccase is a widely used enzyme in many industrial applications. Its capabilities have been used in the degradation of pollutants like polyaromatic hydrocarbons. Directed evolution approach has been used to engineer this enzyme to make it able to degrade 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) much more efficiently than the native one. The laccase gene from *Myceliophthora thermophila* (*MtL*) was transformed into *Saccharomyces cerevisiae* and subjected to 10 rounds of directed evolution which in turn resulted in eightfold increase in its expression with 22-folds in Kcat value (Bulter et al. 2003).

Naphthalene, fluorine, acenaphthalene, acenaphthylene, and 9-methylanthracene are some of the most harmful pollutants which are efficiently degraded by the cytochrome P450 monooxygenases (CYPs). Different research groups have demonstrated the enhancement in the activities of this enzyme by modifying its active site by rational designing. In *Pseudomonas putida,* mutations in CYP101 at F87 and Y96 amplified the oxidation efficiency of these enzymes by threefolds (Harford-Cross et al. 2000), while in *Bacillus megaterium* enzyme CYP102, mutations at R47L and Y51F enhanced the oxidation activity of the enzyme for phenanthrene and fluoranthene with an order of magnitude of 40-folds and tenfolds, respectively (Carmichael and Wong 2001). Triple mutations in CYP at A74G/F87V/L188Q sites enhanced its polyaromatic hydrocarbons degrading ability up to 30-folds with simultaneous increase in NADPH consumption rate when compared to control organism (Liu et al. 2006).

Polychlorinated biphenyls (PCBs) are collectively known as congeners which are consisting of 209 member compounds (Das and Adholeya 2012). Their applications vary from extender in insecticidal to an insulator in transformer production. PCBs are also well-known environment pollutants. Biphenyl dioxygenase enzyme has the affinity to degrade these organic pollutants by aerobic biodegradation (Ang et al. 2005), that is, by the incorporation of two hydroxyl groups into the aromatic ring of a PCB congener. The degradation of PCBs by biphenyl dioxygenase leads to the production of tricarboxylic acid cycle intermediated. This enzyme complex is composed of a terminal dioxygenase (made up of a large α and a small β subunit), ferredoxin, and ferredoxin reductase encoded by the *bph* operon (Erickson and Mondello 1992). By targeting a part of *bphA* taken from various sources like *Burkholderia cepacia* strain LB400, *C. testosteroni* B-365, and *Rhodococcus globerulus* P6, variants were generated with superior PCBs degradation capabilities using family shuffling approach (Barriault et al. 2002). The 2,6-dichlorobiphenyl, which is a very persistent PCB congener, has also been oxygenated by hybrid *BphA*, II-9 genes up to 58% in comparison to controls which were able to oxygenate only up to 10% (Suenaga et al. 2002).

The plasmids pE43 and pPC3, which carry the oxygenolytic *ortho*-dechlorination *ohb* gene and the hydrolytic *para*-dechlorination *fcb* gene, respectively, were used to produce two transformants of PCB-cometabolizing *C. testosteroni* VP44 [VP(pE43) and VP(pPC3)] by individually transforming both the plasmids. The recombinant showed the capability of using *ortho*- and *para*-chlorobiphenyls (CBs) as sole carbon sources up to 95% concentrations of 2- and 4-CBs (Hrywna et al. 1999).

Pseudomonas sp. ADP harbors the genes for the enzymatic hydrolysis of harmful pesticides like 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-*s*-triazine (atrazine). The directed evolution holds promises of accomplishing in the laboratory what nature may not have had time to accomplish in the environment. Atrazine is used for weed control, and it has been reported to have a half-life of around 170 days. *Pseudomonas* sp. produces two forms of atrazine-degrading enzymes which are Atz (A, B, and C) and TriA. It has been observed that these proteins have different mechanism of action, but cyanuric acid

is the final product of degradation. Atz and TriA are enzymes that have likely evolved to degrade atrazine only recently (Wackett 1998).

Paraoxon, parathion, chlorpyrifos, disulfoton, ruelene, carbophenothion, and dimeton are some of the organophosphates which are potent neurotoxins (Das and Adholeya 2012). These compounds prevent the breakdown of acetylcholin esterase at synaptic junction, and there are also reports that these compounds are responsible for chromosomal breakdown and bladder cancer (Webster et al. 2002). Bacterial phosphotriesterase (PTE) and organophosphorus hydrolase (OPH) are the enzymes which are capable to hydrolyze organophosphates by cleavage of P–O, P–F, or P–S bonds. The Sp-enantiomers of organophosphate are generally preferred over the Rp-enantiomers. PTE from *Pseudomonas diminuta* was analyzed for its three-dimensional study with a structural analogue diethyl 4-methylbenzylphosphonate which confirmed three hydrophobic binding pockets for the orientation of substrate in the active site (Chen-Goodspeed et al. 2001a, b). Chen-Goodspeed et al. carried out site-directed mutagenesis at three key sites, that is, Ile106, F132G, and His257. Substitution of Ile106 by Ala resulted in the elimination of 20–90-fold preference for Sp-enantiomers for some chiral substrates, while a combined mutation at I106G/F132G resulted in up to 270 enhancements in Kcat for Rp-enantiomers without any change in the Kcat for Sp-enantiomers. This property is desirable for the bioremediation of racemic mixture of pollutants. And finally, the mutation at third key site by tyrosine resulted in the reduced kinetic parameters of PTE on all tested Sp-enantiomers and thus modification in sterioselectivity (Chen-Goodspeed et al. 2001a). There are some more examples of the genetically modified microorganisms which degrade persistent organic compounds (Table 11.1).

Recombinant Fungi and Bioremediation

Some of the naturally occurring fungi are employed for the degradation or transformation of complex contaminants such as organic pollutants and heavy metals to simpler forms for proper recycling and further uses. However, at many instances, naturally occurring fungi cannot degrade the organic pollutants completely. Enhancement in biological activities of genetically manipulated organisms has attracted considerable attention for bioremediation strategies. Fungal strains can be improved by genetic recombination through interspecific and intraspecific DNA hybridization.

There are several reports on the degradation of environmental pollutants by different fungi (Das and Chandran 2011). Recombinant *Saccharomyces cerevisiae* cells expressing mammalian CYP1A (family of cytochrome P450 complexes) gene are able to degrade dioxins (Sakaki et al. 2002). *Saccharomyces cerevisiae* cells were transformed with expression plasmids containing rat CYP1A1 and rat CYP1A2 with yeast NADPH-P450 reductase to create recombinant strains, higher metabolism of DD, MCDDs, DCDDs, and Tri-CDDs as compared to the naturally occurring microorganisms (Sakaki et al. 2002). However, none of the strain was found suitable to degrade 2,3,7,8-tetra-CDD (most toxic PCDD, polychlorinated dibenzo-*p*-dioxins) (Sakaki et al. 2002). To overcome with this problem, Shinkyo et al. (2003) attempted to make a CYP1A1 enzyme capable of degrading 2,3,7,8-tetra-CDD. It was assumed that CYP1A1 could bind to 2,3,7,8-tetra-CDD if the binding pocket of the enzyme was enlarged, which was developed by the site-directed mutagenesis of modifying rat CYP1A1. Several mutant strains were developed for producing these enzymes. Two mutants (F240A and F240S) showed higher activity towards 2,3,7,8-tetra-CDD degradation (Shinkyo et al. 2003). The modified 2,3,7,8-tetra-CDD metabolizing enzyme could be employed for the bioremediation of dioxin from contaminated sites, dioxin elimination from food items, and clinical treatment for people who accidentally take dioxin into their systems (Shinkyo et al. 2003; Simon 2006).

Naturally occurring *Fusarium solani* present in soil are poor degraders of DDT, and several theories were put forward earlier regarding their poor performance in soil (Rao et al. 1982; Bumpus and Aust 1987). Strains of *Fusarium*

Table 11.1 Persistent organic compounds degrading GEMs

Sr. no.	Organism	Pollutant	Strategy used	Application	References
1.	*E. coli*	Cadmium	Phytochelatin synthase gene from *Schizosaccharomyces pombe* overexpressed in *E. coli* along with Cd transporter Mnt A protein expression	Cd removed from soil and water with high efficiency	Seung et al. (2007)
2.	*Deinococcus radiodurans*	Toluene	*tod* and *xyl* genes of *Pseudomonas putida* were cloned and expressed in *Deinococcus radiodurans* which caused incorporation of carbon from 14C-labelled toluene into cellular macromolecules and carbon dioxide	Toluene and other fuel hydrocarbons occurring together with Cr(VI) and other heavy metals could be degraded frequently	Brim et al. (2006)
3.	*Deinococcus radiodurans*	Mercury	Hg (II) resistance gene (merA) from *Escherichia coli* strain BL308 cloned and expressed in test organism	Reduction of Hg (II) to the less toxic volatile elemental mercury	Brim et al. (1999)
4.	*Burkholderia cepacia*	Organic compounds like toluene	Natural endophyte *of* yellow lupine, *B. cepacia* L.S.2.4 was transformed with toluene-degrading plasmid pTOM from *B. cepacia* G4	Efficient phytoremediation of volatile organic compounds with the help of microorganism	Barac et al. (1999)
5.	*Pseudomonas putida* 06909	Cadmium	Expression of metal-binding peptide (EC20) in this rhizobacterium due to which improved cadmium binding and enhanced cellular toxicity of cadmium	Remediation of mixed metal-organic pollutant sites	Wu et al. (2005)
6.	*Escherichia coli*	Heavy metals	*Arabidopsis thaliana* gene encoding PC synthase (AtPCS) expressed in *Escherichia coli*	Bioremediation and phytoremediation of heavy metals from soil	Sandrine et al. (2003)
7.	*Escherichia coli*	Mercury	The test organism genetically engineered to express mercury transport system and organomercurial lyase enzyme system and overexpressed polyphosphate, a strong chelator of divalent metal ions	Bioremediation of metal ions from soil and water	Kiyono and Hidemitsu (2006)
8.	*Deinococcus radiodurans*	Uranium	*phoN* gene encoding a periplasmic nonspecific acid phosphatase in *Salmonella typhi* was cloned and overexpressed in test organism	Bioremediation of uranium from highly radioactivity polluted sites	Appukuttan et al. (2010)

9.	*Pichia pastoris*	Azo dyes, anthraquinone dyes	*lcc1* cDNA isolated from the white-rot fungus *Trametes trogii* and expressed in *Pichia pastoris* to produce fungal laccase	Bioremediation of various xenobiotic compounds	Maria et al. (2006)
10.	*Nicotiana tabacum*	Cadmium	*MT-I* gene from *Mus musculus* transformed in target plant	Phytoremediation of Cd ions	Pan et al. (1994)
11.	*A. thaliana*	Mercury	*merP* gene from *Bacillus megaterium* transformed in the test plant	Accumulation of Hg ions from soil and water	Hsieh et al. (2009)
12.	*B. juncea*	Selenium	*SMT and APS1* genes from *A. bisulcatus* and *A. thaliana* transformed and expressed in target plant	Hyperaccumulation of Se in plant	LeDuc et al. (2004)
13.	*Populus tremula*	TNT	Target plant was engineered with *Nitroreductase* gene taken from *P. putida*	Explosive pollutant 2,4,6-trinitrotoulene degradation by transgenic plant produced	van Dillewijn et al. (2008)
14.	*Trichoderma atroviride*	Dichlorvos pesticide	Linearized DNA of plasmid pV2 bearing the hygromycin B phosphotransferase (hph) gene was inserted into chromosomes of wild strain T23	Improved dichlorvos degradation capability of the transformed fungus	Tang et al. (2009)
15.	*Gliocladium virens*	Paraoxon and diisopropyl-fluorophosphate	*pCL1* derived from *pJS294* by placing the fungal promoter *(prom1)* from *Cochliobolus heterostrophus* upstream and the *trpC* terminator from *Aspergillus nidulans* downstream of the opd gene was transformed and expressed in target fungus	Degradation of organophosphate pollutants	Dave et al. (1994)
16.	*Fusarium solani*	DDT	Genetically engineered strains with improved dehalogenase activity were raised by parasexual	Superior DDT degradation capability of the recombinant fungus in comparison to parental progeny	Mitra et al. (2001)

solani with improved dehalogenase activity were raised by parasexual hybridization of two such complementary isolates, viz., isolate 1(P–1) (genetically Kel$^+$BenRDBP$^-$Lin$^-$) and 4(P–2) (genetically Kel$^-$BenrDBP$^+$Lin$^+$), showing highest complementation and are compatible for hyphal fusion inducing heterokaryosis. Recombinants with mixed genotype, that is, Kel$^+$BenRDBP$^+$Lin$^+$ showing superior biodegradation capabilities for DDT, were selected for the bioremediation study (Mitra et al. 2001).

Recombinant strain of thermotolerant methylotrophic yeast, *Hansenula polymorpha*, was used for chromate bioremediation, which expressed FC b2 (flavocytochrome b2) enzyme, showing sixfold increased activity compared to the parental strain (Smutok et al. 2011). Flavocytochrome b2 (FC b2) has absolute specificity for L-lactate yet is nonselective with respect to its electron acceptor. These properties are responsible for the activity of this enzyme as a potential biomolecule for chromate reduction by living cells in the presence of L-lactate. In addition, FC b2-overproducing recombinant cells of *H. polymorpha* are used for the construction of cell-based biosensor (Smutok et al. 2011).

Recombinant Algae and Bioremediation

Natural as well as anthropogenic pollution of various marine ecosystems is global environmental issue which requires immediate attention (Bird et al. 1997). Algae are the large group of autotrophic, eukaryotic, and unicellular to multicellular level of organization mostly found in aquatic habitats with more than 20,000 species. The capability to produce large-scale algae populations could possibly provide cost-effective technologies for preventing dissemination and sequestering of waterborne pollutants. These contaminants may include high sewage-based nutrient concentrations, heavy metals, radionuclides, and microbial pathogens (Bird et al. 1997). Algae bioaccumulate a wide variety of heavy metals such as lead, copper, nickel, zinc, cadmium, gold, silver, manganese, tin, chromium, aluminum, and cobalt (Burdin 1985).

Marine algae, particularly brown seaweeds such as *Durvillaea potatorum, Ecklonia radiata*, and *Laminaria japonica*, have much higher adsorption capacity for many heavy metal ions in comparison to the absorption of activated carbon and natural zeolites (Matheickal and Yu 1996; Yu et al. 1998). Marine alga *Tetraselmis suecica* removed cadmium (Perez-Rama et al. 2002), red marine alga *Gracilaria fisheri* was used as the hyperaccumulator of cadmium (II) and copper (II) (Chaisuksant 2003), and marine brown alga *Laminaria japonica* was chemically modified by cross-linking with epichlorohydrin (EC_1, EC_2), or oxidized by potassium permanganate was used to remove Pb from the wastewaters (Luo et al. 2006). Green alga *Chlorella sorokiniana* ANA9 was used for the bioremediation of heavy metals and useful in preventing Cd^{2+} diffusion in to the soil environment (Yoshida et al. 2006).

The microalga *Dunaliella salina* is one of the most tolerant species to cadmium. Nevertheless, it is not a good candidate to be used in processes of cadmium removal from contaminated seawaters (Folgar et al. 2009). Gomes and Asaeda (2009) reported the decreased toxicity of Cr (VI) contaminated in water bodies by employing *Nitella pseudoflabellata.* Algae can evolve various strategies to decrease the toxicity of contaminants of heavy metal environment. The species of *Chlamydomonas* synthesizes heavy metal-binding phytochelatins. Further, to increase the heavy metal-binding capacity of the algae, a foreign metallothionein (MT-II) was expressed in *C. reinhardtii* which is aquatic or lives in damp habitats on land. Cultures that expressed the MT-II were found to be absorbing twofold more Cd as compared to wild-type cultures (Cai et al. 1997; Perales-Vela et al. 2006).

Recombinants in Environmental Pollution Monitoring

The growing awareness about the impacts of environmental pollution has triggered a number of global remediation actions in recent years. In order to apply a technique to mitigate the effect of a pollutant on the environment, the first requirement is to have a fair idea about the presence of a

particular target pollutant. Although there are numerous traditional methods for pollutant monitoring, for example, to monitor heavy metals such as cadmium (Cd), zinc (Zn), lead (Pb), arsenic (As), and copper (Cu) in the territory of Poland and the Warsaw (Warszawa) district, the chemical analysis of the Scots pine (*Pinus sylvestris* L.) needles was done by collecting the needles from randomly chosen sites (Wojciech and Andrzej 1995). In another report, the bark of *Quercus robur* and *Quercus petraea* was used for determining the distribution of air pollution by heavy metals in the western part of the Czech Republic. The samples were analyzed using instrumental neutron activation analysis (INAA) and radionuclide X-ray fluorescence analysis (RXRFA) for different heavy metals (Böhm et al. 1998).

The drawbacks of these approaches are time consumption and efforts with utilization of large amount of financial input. These approaches also fail to provide information on the bioavailability of the analyzed compounds or their effects on biological systems. So, to circumvent these issues, more reliable and cost-effective approaches are being used. The use of biosensors and the deployment of bioluminescent microorganisms for the monitoring of pollution are two different technologies. Mulchandani et al. (1998) developed a biosensor for the detection of organophosphorus by immobilizing recombinant *E. coli* which expressed organophosphorus hydrolase on the cell surface. The reaction mechanism of the detection of the target molecule was based upon the optical transduction system as the signal produced by the hydrolysis of organophosphorus molecules. This detection system was cost effective in the sense that it obviates the need of the purified enzyme. Also the presence of the enzyme molecules on the surface of the microorganism neglected the problem of inhibition by mass transfer. In another approach by Kumar and D'Souza, a whole-cell biosensor was developed to detect organophosphorus pesticides using the *Sphingomonas* sp. This organism produces enzyme that hydrolyzes the methyl parathion to a chromophoric product, p-nitrophenol (PNP). This chromophoric product is detected using electrochemical and colorimetric methods, and the extent of the composition of pesticides in the soil could be observed (Kumar and D'Souza 2010).

Monitoring of environmental changes has been facilitated using reporter genes fused to promoters that respond to specific environmental pollutants (Paitan et al. 2004). As the promoters are capable to regulate the expression of a gene under the influence of physical parameters like temperature, pH, and ionic concentration as well as under the effect of repressor or enhancer so, it is possible to have an idea about the environmental concentration of a pollutant by measuring the activity of the promoter. A sensing element and a reporter gene are the essential components of a microbial cell which is tamed to function as microbial bioreporter. A sensing element is generally a promoter or a group of genes activated in presence of an environmental signal. Normally, when the sensing element gets activated, it produces the protein by the action of which the hazardous condition is sensed, while in the recombinant strain, in addition to this function, the selected promoter also drives the synthesis of the reporter protein(s) (Köhler et al. 2000).

Bacterial β-galactosidase, luciferase, β-glucuronidase (GUS), alkaline phosphatase, and β-lactamase are some of the most studied reporter proteins. The specificity of all these proteins lies in the sense that they all are either readily detectable or are capable of an easily measured activity. The assembly of promoter and reporter genes can be established in the bacterial cell either in a plasmid or can be permanently incorporated in chromosome; however, the latter strategy is lengthier but more stable (Köhler et al. 2000). Recombinant *Pseudomonas fluorescens* strain HK44 was the first field-released GEM for the bioremediation of polyaromatic hydrocarbons (PAHs). This strain was transformed with naphthalene catabolic plasmid pUTK21. As the modified strain also contains a transposon-based bioluminescence-producing *lux* gene fused within a promoter for naphthalene catabolic gene, so the exposure of the recombinant strain to naphthalene resulted in the increased catabolic gene expression, naphthalene degradation, and simultaneous bioluminescent response (King et al. 1990; Sayler et al. 1999; Ripp et al. 2000).

Microbial Cell-Surface Display Engineering and Bioremediation

Cell-surface display of heterologous proteins by genetic engineering has emerged as a novel and economic approach for the bioremediation of various organic and inorganic contaminants (Saleem et al. 2008). In this approach, the functional proteins are expressed on the surface of the microbial cell through a translational fusion with the naturally occurring target protein, which endows intact cells with new functionalities (Ueda and Tanaka 2000a, b). There are many reports on the heterologous surface expression of proteins for the enhanced bioremediation of different contaminants such as heavy metals, xenobiotics, and rare metals (Chen and Georgiou 2002; Dong et al. 2006; Narita et al. 2006; Kuroda and Ueda 2010, 2011).

Many gram-positive and gram-negative bacteria have been reported to be engineered for the display of various heavy metal-binding proteins such as phytochelatins, metallothioneins, hexahistidine, mercury-responsive metalloregulatory protein, cysteine-rich peptide (Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly, CP), histidine-rich peptide (Gly-His-His-Pro-His-Gly, HP), and bioadsorbents, for the bioremediation of heavy metal from contaminated sites (Sousa et al. 1998; Kotrba et al. 1999; Bae et al. 2001, 2003; Samuelson et al. 2000; Kuroda et al. 2001; Kuroda and Ueda 2010). Adsorption of several heavy metals such as cadmium, nickel, and copper by genetically engineered yeast cells displaying metal-binding proteins has already been reported (Fig. 11.5). Genetically engineered yeast cells have been found to tolerate a higher level of heavy metal concentration as compared to wild-type strain (Kuroda et al. 2001). The engineering of microorganisms for protein surface expression may also functions to alter the cellular metabolism to make the cell survive at the higher concentration of heavy metals (Kuroda and Ueda 2011). Takayama et al. (2006) reported the expression of hydrolase (OPH) from *Flavobacterium* species on the surface of *Saccharomyces cerevisiae* cells for detoxification of organophosphorus compounds.

Anticipated Risks with Genetically Engineered Microorganisms

No doubt, the deployment of the recombinant DNA technology for the production of GEMs and to use these modified microorganisms is a very cost-effective way of neutralizing the environmental pollutant. If the reports of last decade are analyzed, a negligible number of transgenic microorganisms have been used in the field; in fact no true field releases have been performed. An increased interest in the development of transgenic microorganisms for different environmental applications during 1980s has ignited an ethical argument related to possible risks of using the genetically modified microorganisms in open (Singh et al. 2011).

The slow progress in the GEMs release is attributed to some safety concerns, legislative constraints, and potential risks perceived by the general public which results in tight regulation (Paul et al. 2005). United Nations Environmental Programme (UNEP) and the Organization for Economic Co-operation and Development (OECD) are two international organizations which are keeping close eyes on the regulation of genetically modified organisms (GMOs) or living modified organisms (LMOs) (OECD 1992, 1994). Generally, the experimental work in the risk assessment does not always fit in all the norms provided by regulatory agencies. It has been suggested by the regulatory agencies that before the release of a mutated microorganism, its genetic fate has to be determined in comparison to the wild-type strain in the containment system with all the necessary field conditions. Localization of the heterologous gene in the host genome must have to be determined, and the transfer of this element should not occur at large extent (Smith et al. 1992). Other than above-mentioned difficulties, the determination of the possible impacts on the ecosystem diversity and function is the most challenging process. As a whole, we can say that the main concern is the designing of GE microorganism for field release in bioremediation with an adequate affinity of environmental certainty.

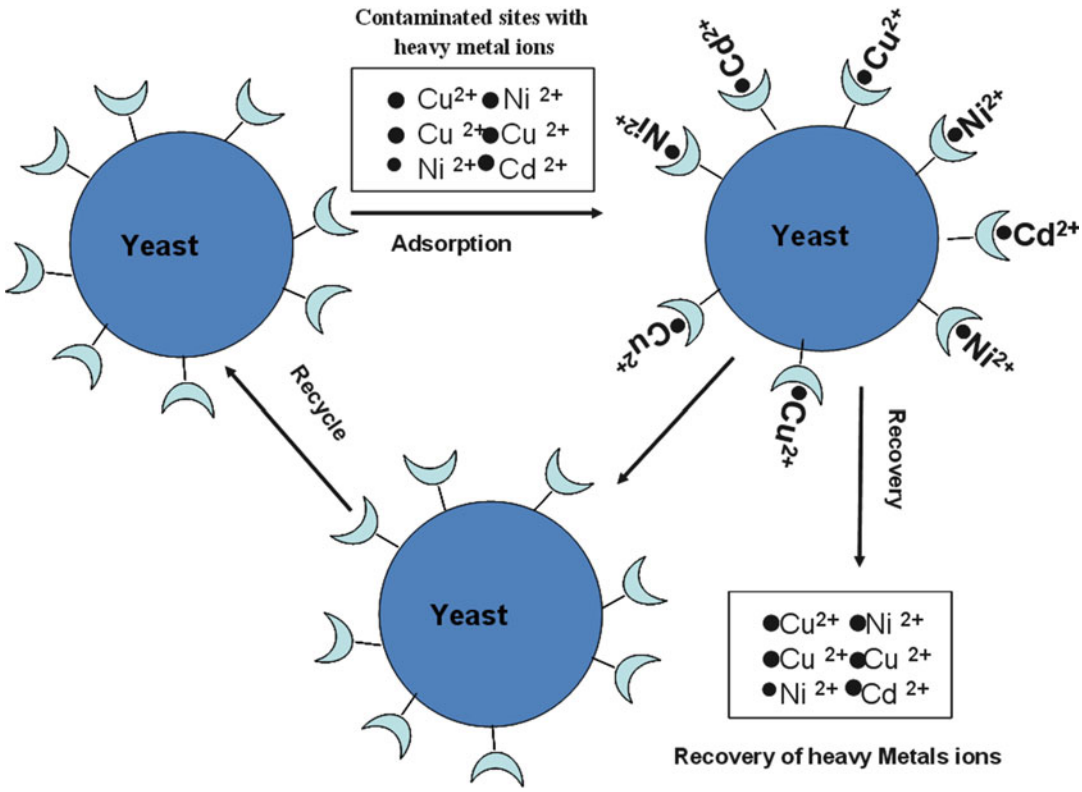

Fig. 11.5 Yeast cell-surface display technology and bioremediation (Modified from Kuroda and Ueda 2011)

There are reports on the harmful impacts of many microorganisms that are commonly being used as ideal bioremediation agents. *Burkholderia cepacia* which has significant capabilities to bioremediate harmful nitro compounds is a pathogen for cystic fibrosis disease in humans, and also this organism shows multiple resistances to antibiotics. Environmental Protection Agency has led to the inhibition of its use as environmental protection agent. Toluene degradation by *Pseudomonads* is also accomplished by unregulated functioning of efflux pumps, and some of the pumps are responsible for the pumping out of various antibiotics and biocides. Thus, the effect of selecting this organism as environment remediator may be accomplished by the production of antibiotic resistance progeny (Davison 2005).

Horizontal transfer of recombinant genes from the host organism to the other microorganisms in its neighborhood is spontaneous process that occurs by transduction, conjugation, or by other means. Even the interspecies gene transfer has also been observed for many specific bacteria at different ecological conditions (Maria et al. 2011). The analysis of the prokaryotic genome has revealed that the horizontal gene transfer continues to be an important factor contributing to the innovation of the bacterial genome (Patricia and Jonna 2009). This type of the transfer of genetic information between bacterial worlds is a potential hurdle in the development of genetically engineered microbial systems. However, if suitable techniques are applied during designing a bacterial system for bioremediation, the possibilities of the reduction of these issues can be increased up to a great extent.

If the environment safety is a concern, the construction of transposition vectors is required which do not harbor antibiotic resistance genes as antibiotic vectors are undesirable for environmental safety. Bacterial containment systems for designing suicidal genetically engineered microorganisms

are one of the most promising strategies as far as mitigation of the risks associated with environmental release of recombinant microorganisms is concerned. This approach is discussed in detail in next section.

Suicidal Genetically Engineered Microorganisms (S-GEMs)

There can be two fates of the genetically engineered microorganisms: the preferred one is the completion of the prescribed work by the organism and then its complete elimination from the environment. The alternative way, which is generally not supported, is that the organism will not be eliminated but will survive and proliferate. The first option is the best suited as if the recombinant microorganisms will remain in the environment, there may arise undesirable effects in the ecosystems (Paul et al. 2005). So, to minimize the undesirable affects in the environment, the survival ability of the strains should be limited by the construction of special containment systems. Some of the systems that have been developed by various research groups are discussed in detail.

Plasmid Addiction System

In case of bacterial plasmids, which possess a high degree of stability even in the absence of selective pressure, it has been reported that even the low copy-number plasmids are properly distributed and maintained in bacterial cells (Kues and Stahl 1989; Thomas 2000). Plasmid addiction system is the technique first devised by Koyama et al. in 1975 in order to regulate the discrepancy in plasmid distribution (Koyama et al. 1975). This approach is based upon the principle that any bacterial cell would not be capable to survive if it loses the established plasmid. Various nomenclatures have been given to this system according to the type of proteins involved in the process such as toxin-antitoxin, poison-antidote, post-segregation killing, or programmed cell death. Generally, this system contains at least two genes, one specifying for a stable toxin which is certainly a protein and another gene is for the production of a factor (protein or antisense RNA) that provides resistance to the cell against this toxin (Paul et al. 2005). Functioning of this system is based on the differential decay rate of both the products, that is, killer protein is in excess and more stable than its rival factor. The survival ability of the cell depends upon the presence of the antidote factor. As long as the cell bears its plasmid, the cell death is prevented. In plasmid-free cells, the concentration of the antidote quickly falls, and the toxic action of lethal protein causes cell death (Pieper and Reineke 2000) (Fig. 11.6).

The killing mechanism of toxin is not well explored; in fact only a few techniques are characterized completely. Liu et al. (2008) determined the mode of action of the Doc toxin of the Phd-Doc toxin-antitoxin system. In this report, it was found that Doc did not cleave mRNA as do other addiction-module toxins whose activities result in translation inhibition. Instead, the Doc induction mechanism showed interaction with 30S ribosomal subunits, stabilized polysomes, and resulted in a significant increase in mRNA half-life. In this sense, Doc was mimicking the action of hygromycin B. In another report, killing mechanism of action of Ccd, PemK, and Kid toxins was revealed, which are encoded by *ccd* and *pem/parD* located on plasmids F and R100/R1, respectively. CcdB toxin inhibits the gyrase by forming a complex with the free GyrA subunit. However, CcdA antidote prevents the formation of this complex and also releases CcdB by forming inactive complexes, that is, CcdA-CcdB (Mario et al. 2009). The PemK and Kid toxins act on DnaB helicase and inactivate replication. The replication is restored when cognate antitoxins are present in the cell (Jensen et al. 1995).

Use of antisense RNA instead of protein as antidote factor has been encouraged in some reports. As an example, the three genes system, that is, *hok (host killing), sok (suppressor of killing), and mok (modulation of killing)* located on *parB* locus of plasmid R1, has been used as containment strategy. The product of *hok* killer gene which is a hydrophobic peptide of 52 amino acids associated with the cellular membrane is lethal to the cell when

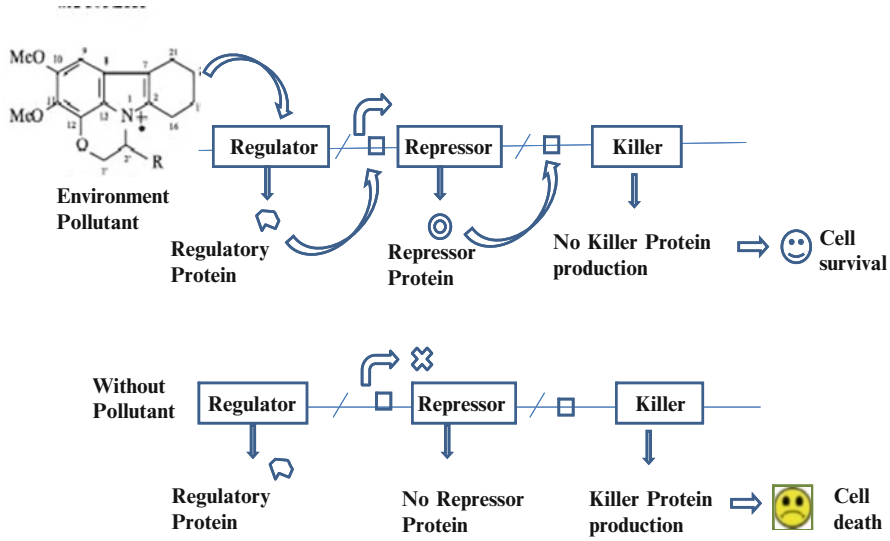

Fig. 11.6 Plasmid addiction system

overexpressed. The product of *mok* gene is required for the expression and regulation of *hok* translation. In this system, *sok* encodes an antisense RNA which is complementary to *hok* mRNA leader region and acts as antidote. Again the difference in the decay rate of *hok* and *sok* RNA is the basis of this plasmid addiction system (Paul et al. 2005).

Other Mechanisms of Containment Systems

The killer gene and the regulatory circuit are the two key parts of a bacterial containment system. The regulatory circuits contain inducible promoters and regulators that control the activity of the promoters in the presence of suitable effector molecules. The expression of the killer gene is controlled by the regulatory circuit in response to the environmental signals. Several containment systems have been developed by incorporating promoters such as *Pm, Ptrp,* and *Ptac* and transcription regulators such as those belonging to the LysR and AraC families (Gallegos et al. 1997). Nonspecific nature of these transcriptional regulators could facilitate their use in construction of containment systems that respond to a variety of substrates (Paul et al. 2005).

One of the simplest examples of the suicide mechanisms is regulation of the cell survival by the presence or absence of the environmental signals, that is, the pollutant against which the system was developed. As a best investigated example, the lethal *gef* gene is carried by a mini-Tn5 transposon, placed under the control of *LacI* repressor. In this assembly, transcription of the *lacI* gene itself depends on the XylS positive activator, which is active only in the presence of 3-methylbenzoate. Thus, the cell remains viable as long as the signal of 3-methylbenzoate is present. As this chemical signal becomes depleted, may be due to degradation or due to escape in the environment, the cell gets killed by the action of lethal gene *gef* (Davison 2002; Torres et al. 2003).

The containment systems have one disadvantage where the effect of the lethal gene gets lost due to mutation in some cases. To be on safer side, doubly contained strains have been designed. One defined example is based on the *asd* gene for diaminopimelic acid synthesis which is essential for cell wall synthesis (Qin and Wu 2009). *Pseudomonas putida* carrying a deletion of the *asd* gene was provided with an external *asd* gene under the positive control of XylS activator, the same activator which also negatively controls *gef*

expression as described above. Thus, a strain deprived of 3-methylbenzoate would die due to killing by Gef protein and also due to diaminopimelic acid deprivation (Ronchel and Ramos 2001). All the above-mentioned suicide mechanisms provide a reliable system to prohibit the horizontal transfer of genes between bacteria via various sexual means. Alternatively few more approaches have been developed based upon the protein colicin E3 encoded by *colE3* gene which cleaves 16S rRNA and kills the bacterial cell. Normally, the killing effect of this gene is neutralized by the antidote effect by an immunity function which is encoded by *immE3* gene. In one approach, *colE3* gene was clone in the plasmid, and *immE3* was placed in the chromosome of *E. coli*. It was observed that the transfer of the plasmid to other cells resulted in the death of the recipient (Torres et al. 2003). To circumvent the problem related to mutation in the killing function, a second approach was used in which two independent systems were designed, both of which were based on two different toxins, the killing mechanism of which was different and mutation in one system could not be affective for the second. Two lethal genes, *colE3* for colE3 toxin and *ecoRIR* for *Eco*RI restriction endonucleases, were cloned in different plasmid, and the gene coding for antidotes (*immE3* and *ecoRM*) against the toxins was placed on the bacterial genome. This system showed superior properties compared to single containment approach (Torres et al. 2003).

Conclusion and Future Challenges

Designing a recombinant microorganism according to the hostile field condition is the main limiting factor governing the success profile in the area of bioremediation. Particularly, both technical and ethical obstacles lead to constraints to the effective release of GEMs. However, with the recent advancements in the biomolecular engineering, it is possible to shorten the process of natural evolution to degrade xenobiotic pollutants. It can be concluded from the above-discussed literature that despite various advancements in the field of the development of pollution monitoring system using microbial biosensors, there are certain limitations in their uses. Large variability in sensitivity, response times, detection thresholds, signal relaxation lengths, and stability is a major obstacle. In some cases, there is also a deficit of information concerned with the functionality of the microbial biosensors in "real" samples to which they may be subjected. More dedicated efforts are needed in order to understand the molecular control of the designed system. Also, the identification of the factors that enhance the in situ bioremediation by engineered bacteria could be a target for the future research.

Moreover, uncontrolled growth of the GEMs and their high potential to spread new genetic information to recipients is a major obstacle in the validation of a recombinant under field conditions. Therefore, designing of novel bacteria suicidal systems in order to control the growth and prevention of horizontal transfer of genes and therefore to avoid the appearance of undesirable genetic traits can provide the further direction in developing novel recombinants.

References

Amin N, Liu AD, Ramer S, Aehle W, Meijer D, Metin M, Wong S, Gualfetti P, Schellenberger V (2004) Construction of stabilized proteins by combinatorial consensus mutagenesis. Protein Eng Des Sel 17:787–793

Anderson KL, Dunman PM (2009) Messenger RNA turnover processes in *Escherichia coli, Bacillus subtilis*, and emerging studies in *Staphylococcus aureus*. Int J Microbiol 2009:15 p, Article ID 525491

Ang EL, Zhao H, Obbard JP (2005) Recent advances in the bioremediation of persistent organic pollutants via biomolecular engineering. Enzyme Microb Technol 37:487–496

Ang EL, Obbard JP, Zhao HM (2007) Probing the molecular determinants of aniline dioxygenase substrate specificity by saturation mutagenesis. FEBS J 274:928–939

Ang EL, Obbard JP, Zhao H (2009) Directed evolution of aniline dioxygenase for enhanced bioremediation of aromatic amines. Appl Microbiol Biotechnol 81:1063–1070

Appukuttan D, Chitra SN, Padma A, Sambasiva R, Shree KA (2010) PhoN-expressing, lyophilized, recombinant *Deinococcus radiodurans* cells for uranium bioprecipitation. J Biotechnol 154:185–190

Armengaud J, Happe B, Timmis KN (1998) Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J Bacteriol 180:3954–3966

Attila C, Ueda A, Cirillo SLG, Cirillo JD, Chen W, Wood TK (2008) *Pseudomonas aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere. Microb Biotechnol 1:17–29

Bae W, Mehra R, Mulchandani A, Chen W (2001) Genetic engineering of *Escherichia coli* for enhanced bioaccumulation of mercury. Appl Environ Microbiol 67:5335–5338

Bae W, Wu CH, Kostal J, Mulchandani A, Chen W (2003) Enhanced mercury biosorption by bacterial cells with surface-displayed MerR. Appl Environ Microbiol 69:3176–3180

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, van der Lelie D (1999) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nat Biotechnol 22(5):583–588

Barkay T, Susan MM, Summers AO (2003) Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol Rev 27:335–384

Barriault D, Plante MM, Sylvestre M (2002) Family shuffling of a targeted *bphA* region to engineer biphenyl dioxygenase. J Bacteriol 184:3794–3800

Bird KT, Bourdine KS, Busch WS (1997) Marine algae as a tool for bioremediation of marine ecosystems. Agric Biotechnol (Arie Altman, CRC Press) 29:601–613

Böhm P, Wolterbeek H, Verburg T, Musílek L (1998) The use of tree bark for environmental pollution monitoring in the Czech Republic. Environ Pollut 102:243–250

Bosma T, Damborsky J, Stucki G, Janssen DB (2002) Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase gene. Appl Environ Microbiol 68:3582–3587

Brim H, Heyndrickx M, De Vos P, Wilmotte A, Springael D, Schlegel HG, Mergeay M (1999) Amplified rDNA restriction analysis and further genotypic characterisation of metal-resistant soil bacteria and related facultative hydrogenotrophs. Syst Appl Microbiol 22(2):258–268

Brim H, Osborne JP, Kostandarithes HM, Fredrickson JK, Wackett LP, Daly MJ (2006) *Deinococcus radiodurans* engineered for complete toluene degradation facilitates Cr(VI) reduction. Microbiology 152:2469–2477

Bulter T, Alcalde M, Sieber V, Meinhold P, Schlachtbauer C, Arnold FH (2003) Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. Appl Environ Microbiol 69:987–995

Bumpus JA, Aust SD (1987) Biodegradation of DDT [1,1,1- trichoro-2,2_ -bis (4_-chlorophenyl) ethane] by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 53:2001–2008

Burdin KS (1985) The basis of biological monitoring, vol 160. Moscow State University Press, Moscow

Burlage RS, Hooper SW, Sayler GS (1989) The TOL (pWWO) catabolic plasmid. Appl Environ Microbiol 55(6):1323–1328

Cai XH, Adhiya J, Brown C, Traina S, Sayre R (1997) Heavy metal binding properties of wild type and transgenic algae. Plant Biol 114(3):555

Carlos G, Alkorta I (1999) Utilization of genetically engineered microorganisms (GEMs) for bioremediation. J Chem Technol Biotechnol 74:599–606

Carmichael AB, Wong LL (2001) Protein engineering of *Bacillus megaterium* CYP102—the oxidation of polycyclic aromatic hydrocarbons. Eur J Biochem 268:3117–3125

Carrier TA, Keasling JD (1997) Controlling messenger RNA stability in bacteria: strategies for engineering gene expression. Biotechnol Prog 13:699–708

Chaisuksant Y (2003) Biosorption of cadmium (II) and copper (II) by pretreated biomass of marine alga *Gracilaria fisheri*. Environ Technol 24:1501–1508

Chatterjee R, Yuan L (2006) Directed evolution of metabolic pathways. Trends Biotechnol 24:28–38

Chauhan A, Fazlurrahman, Oakeshott OG, Jain RK (2008) Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. Indian J Microbiol 48:95–113

Chen W, Georgiou G (2002) Cell-Surface display of heterologous proteins: from high-throughput screening to environmental applications. Biotechnol Bioeng 79:496–503

Chen W, Mulchandani A (1998) The use of live biocatalysts for pesticide detoxification. Trends Biotechnol 16:71–76

Chen-Goodspeed M, Sogorb MA, Wu FY, Raushel FM (2001a) Enhancement, relaxation, and reversal of the stereo selectivity for phosphotriesterase by rational evolution of active site residues. Biochemistry 40:1332

Chen-Goodspeed M, Sogorb MA, Wu FY, Hong SB, Raushel FM (2001b) Structural determinants of the substrate and stereochemical specificity of phosphotriesterase. Biochemistry 40:1325

Dai M, Copley SD (2004) Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. Appl Environ Microbiol 70:2391–2397

Das M, Adholeya A (2012) Role of microorganisms in remediation of contaminated soil. Microorg Environ Manag 81–112

Das N, Chandran P (2011) Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Int 2011, 941810

Dave KI, Lauriano C, Xu B, Wild JR, Kenerley CM (1994) Expression of organophosphate hydrolase in the filamentous fungus *Gliocladium virens*. Appl Microbiol Biotechnol 41:352–358

Davison J (2002) Genetic tools for Pseudomonads, Rhizobia and other gram-negative bacteria. Biotechniques 32:386–401

Davison J (2005) Risk mitigation of genetically modified bacteria and plants designed for biodegradation. J Ind Microbiol Biotechnol 32:639–650

de Lorenzo V (1994) Designing microbial systems for gene expression in the field. Tib Tech 12(9):365–371

de Lorenzo V (2009) Recombinant bacteria for environmental release: what went wrong and what we have learnt from it. Clin Microbiol Infect 15:63–65

de Lorenzo V, Fernandez S, Herrero M, Jakubzik U, Timmis KN (1993) Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. Gene 130:41–46

DeLong EF, Pace NR (2001) Environmental diversity of bacteria and archaea. Syst Biol 50:470–478

Deng X, Wilson DB (2001) Bioaccumulation of mercury from wastewater by genetically engineered *Escherichia coli*. Appl Microbiol Biotechnol 56:276–279

Deng X, Yi XE, Liu G (2007) Cadmium removal from aqueous solution by gene modified *Escherichia coli* JM109. J Hazard Mater 139:340–344

Dong J, Liu C, Zhang J, Xin ZT, Yang G, Gao B, Mao CQ, Liu NL, Wang F, Shao NS, Fan M, Xue YN (2006) Selection of novel nickel binding peptides from flagella displayed secondary peptide library. Chem Biol Drug Des 68:107–112

Endo G, Silver S (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J Bacteriol 177:4437–4441

Erickson BD, Mondello FJ (1992) Nucleotide sequencing transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain-LB400. J Bacteriol 174:2903–2912

Ezezika OC, Singer PA (2010) Genetically engineered oil-eating microbes for bioremediation: prospects and regulatory challenges. Technol Soc 32:331–335

Fetzner S (2007) Cofactor-independent oxygenases go it alone. Nat Chem Biol 3(7):374–375

Folgar S, Torres E, Pérez-Rama M, Cid A, Herrero C, Abalde J (2009) *Dunaliella salina* as marine microalga highly tolerant to but a poor remover of cadmium. J Hazard Mater 165:486–493

Fuenmayor SL, Wild M, Boyes AL, Williams PA (1998) A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J Bacteriol 180:2522–2530

Gallegos MT, Schleif R, Bairoch A, Hofmann K, Ramos JL (1997) AraC/XylS family of transcriptional regulators. Microbiol Mol Biol Rev 61:393–410

Gallie D, Kado C (1989) Proc Natl Acad Sci USA 86:129–132

Gao H, Wang Y, Liu X, Yan T, Wu L, Alm E, Arkin A, Dorothea KT, Zhou J (2004) Global Transcriptome analysis of the heat shock response of *Shewanella oneidensis*. J Bacteriol 18:7796–7803

Gomes PL, Asaeda T (2009) Phycoremediation of Chromium (VI) by *Nitella* and impact of calcium encrustation. J Hazard Mater 166(2–3):1332–1338

Hamamatsu N, Aita T, Nomiya Y, Uchiyama H, Nakajima M, Husimi Y, Shibanaka Y (2005) Biased mutation-assembling: an efficient method for rapid directed evolution through simultaneous mutation accumulation. Protein Eng Des Sel 18:265–271

Harford-Cross CF, Carmichael AB, Allan FK, England PA, Rouch DA, Wong LL (2000) Protein engineering of cytochrome P450(cam) (CYP101) for the oxidation of polycyclic aromatic hydrocarbons. Protein Eng 13:121–128

Haro MA, deLorenzo V (2001) Metabolic engineering of bacteria for environmental applications: construction of *Pseudomonas* strains for biodegradation of 2-chlorotoluene. J Biotechnol 13:103–113

Hibbert EG, Dalby PA (2005) Directed evolution strategies for improved enzymatic performance. Microb Cell Fact 4:29

Hibbert EG, Baganz F, Hailes HC, Ward JM, Lye GJ, Woodley JM, Dalby PA (2005) Directed evolution of biocatalytic processes. Biomol Eng 22:11–19

Holtwick R, von Wallbrunn A, Keweloh H, Meinhardt F (2001) A novel rolling-circle-replicating plasmid from *Pseudomonas putida* P8: molecular characterization and use as vector. Microbiology 147: 337–344

Hrywna Y, Tsoi TV, Maltseva OV, Quensen JF, Tiedje JM (1999) Construction and characterization of two recombinant bacteria that grow on *ortho*- and *para*-substituted chlorobiphenyls. Appl Environ Microbiol 65:2163–2169

Hsieh JL, Chen CY, Chiu MH, Chein MF, Chang JS, Endo G, Huang CC (2009) Expressing a bacterial mercuric ion binding protein in plant for phytoremediation of heavy metals. J Hazard Mater 161:920–925

Izmalkova TY, Sazonova OI, Sokolov SL, Kosheleva IA, Boronin AM (2005) The P-7 incompatibility group plasmids responsible for biodegradation of naphthalene and salicylate in fluorescent Pseudomonads. Microbiology (Pleiades Publishing) 74:342–348

Jensen RB, Grohmann E, Schwab H, Diaz-Orejas R, Gerdes K (1995) Comparison of ccd of F, parDE of RP4, and par D of R1 using a novel conditional replication control system of plasmid R1. Mol Microbiol 17:211–220

Kalyaeva E, Bass I, Kholodii G, Nikiforov V (2002) A broad host range plasmid vector that does not encode replication proteins. FEMS Microbiol Lett 211:91–95

Kapley A, Purohit HJ (2009) Genomic tools in bioremediation. Indian J Microbiol 49:108–113

Keasling JD, Bang SW (1998) Recombinant DNA techniques for bioremediation and environmentally-friendly synthesis. Curr Opin Biotechnol 9:135–140

Keenan BG, Leungsakul T, Barth FS, Thomas KW (2004) Saturation Mutagenesis of *Burkholderia cepacia* R34 2,4-Dinitrotoluene Dioxygenase at DntAc Valine 350 for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone. Appl Environ Microbiol 70:3222–3231

Kellner DG, Maves SA, Sligar SG (1997) Engineering cytochrome P450s for bioremediation. Curr Opin Biotechnol 8:274–278

King JMH, DiGrazia PM, Applegate B, Burlage RS, Sanseverino J, Dunbar P, Larimer F, Sayler GS (1990) Rapid, sensitive bioluminescence reporter technology for naphthalene exposure and biodegradation. Science 249:778–781

Kiyono M, Hidemitsu PH (2006) Genetic engineering of bacteria for environmental remediation of mercury. J Health Sci 52:199–204

Kiyono M, Sone Y, Nakamura R, Pan-Hou H, Sakabe K (2009) The Mer E protein encoded by transposon Tn21 is a broad mercury transporter in *Escherichia coli*. FEBS Lett 583:1127–1131

Köhler S, Belkin S, Schmid RD (2000) Reporter gene bioassays in environmental analysis. Fresenius J Anal Chem 366:769–779

Kolatka K, Kubik S, Rajewska M, Konieczny I (2010) Replication and partitioning of the broad-host-range plasmid RK2. Plasmid 64:119–134

Kostal JRY, Wu CH, Mulchandani A, Chen W (2004) Enhanced arsenic accumulation in engineered bacterial cells expressing ArsR. Appl Environ Microbiol 70:4582–4587

Kotrba P, Doleckova L, deLorenzo V, Ruml T (1999) Enhanced bioaccumulation of heavy metal ions by bacterial cells due to surface display of short metal binding peptides. Appl Environ Microbiol 65:1092–1098

Koyama AH, Wada C, Nagata T, Yura T (1975) Indirect selection for plasmid mutants: isolation of colVBtrp mutants defective in self-maintenance in *E. coli*. J Bacteriol 122:73–79

Kues U, Stahl U (1989) Replication of plasmids in gram-negative bacteria. Microbiol Rev 53:491–516

Kumamaru T, Suenaga H, Mitsuoka M, Watanabe T, Furukawa K (1998) Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. Nat Biotechnol 16:663–666

Kumar J, D'Souza SF (2010) An optical microbial biosensor for detection of methyl parathion using *Sphingomonas* sp. immobilized on microplate as a reusable biocomponent. Biosens Bioelectron 26:1292–1296

Kuroda K, Ueda M (2010) Engineering of microorganisms towards recovery of rare metal ions. Appl Microbiol Biotechnol 87:53–60

Kuroda K, Ueda M (2011) Cell surface engineering of yeast for applications in white biotechnology. Biotechnol Lett 33:1–9

Kuroda K, Shibasaki S, Ueda M, Tanaka A (2001) Cell surface-engineered yeast displaying a histidine oligopeptide (hexa-His) has enhanced adsorption of and tolerance to heavy metal ions. Appl Microbiol Biotechnol 57:697–701

LeDuc DL, Tarun AS, Montes BM, Meija J, Malit M, Wu CP, Abdel SM, Chiang CY, Tagmount A, de Souza MP, Neuhierl B, Böck A, Caruso J, Terry N (2004) Overexpression of selenocysteine methyltransferase in Arabidopsis and Indian mustard increases selenium tolerance and accumulation. Plant Physiol 135:377–383

Lee J, Cao L, Ow SY, Martin EBL, Chen W, Wood TK, Wright PC (2006) Proteome changes after metabolic engineering to enhance aerobic mineralization of cis-1,2-dichloroethylene. J Proteome Res 5:1388–1397

Leungsakul T, Johnson GR, Wood TK (2006) Protein engineering of the 4-methyl-5-nitrocatechol monooxygenase from *Burkholderia* sp. strain DNT for enhanced degradation of nitroaromatics. Appl Environ Microbiol 72:3933–3939

Liu Z, Hong Q, Xu JH, Jun W, Li SP (2006) Construction of a genetically engineered microorganism for degrading organophosphate and carbamate pesticides. Int Biodeter Biodegr 58:65–69

Liu M, Zhang Y, Inouye M, Woychik NA (2008) Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. Proc Natl Acad Sci USA 105(15): 5885–5890

Liu S, Zhang F, Chen J, Sun GX (2011) Arsenic removal from contaminated soil via biovolatilization by genetically engineered bacteria under laboratory conditions. J Environ Sci 23(9):1544–1550

Luo F, Liu Y, Li X, Xuan Z, Ma J (2006) Biosorption of lead ion by chemically-modified biomass of marine brown algae *Laminaria japonica*. Chemosphere 64:1122–1127

Maria CC, Lupino S, Garzillo AM, Buonocore V, Ruzzi M (2006) Heterologous expression of *lcc1* gene from *Trametes trogii* in *Pichia pastoris* and characterization of the recombinant enzyme. Microb Cell Fact 5:31

Maria FVT, Oscar CBR, Salazar C, Martha JVF, Dussan J (2011) Horizontal arsC gene transfer among microorganisms isolated from arsenic polluted soil. Int Biodeter Biodegr 65:147–152

Mario S, Natalie DJ, Remy L, Vesnave G, Lah J (2009) Driving forces of gyrase recognition by the addiction toxin CcdB. J Biol Chem 284:20002–20010

Marwick C (2003) Link found between Agent Orange and chronic lymphocytic leukaemia. BMJ (Clin Res Ed) 326(7383):242

Matheickal JT, Yu Q (1996) Biosorption of lead from aqueous solutions by marine algae *Ecklonia radiata*. Water Sci Technol 34:1–7

Mattozzi MP, Sundiep KT, Thomas H, Keasling JD (2006) Mineralization of paraoxon and its use as a sole C and P source by a rationally designed catabolic pathway in *Pseudomonas putida*. Appl Environ Microbiol 72(10):6699–6706

Mitra J, Mukherjee PK, Kale SP, Murthy NBK (2001) Bioremediation of DDT in soil by genetically improved strains of soil fungus *Fusarium solani*. Biodegradation 12:235–245

Moharikar A, Kapley A, Purohit HJ (2003) Detection of dioxygenase genes present in various activated sludge. Environ Sci Pollut Res 10:373–378

Morley KL, Kazlauskas RJ (2005) Improving enzyme properties: when are closer mutations better? Trends Biotechnol 23:231–237

Mulchandani A, Irina K, Wilfred C (1998) Surface-expressed organophosphorus hydrolase-2. fiber-optic microbial biosensor. Ann Chem 70:5042–5046

Narita J, Okano K, Tateno T, Tanino T, Sewaki T, Sung MH, Fukuda H, Kondo A (2006) Display of active enzymes on the cell surface of *Escherichia coli* using PgsA anchor protein and their application to bioconversion. Appl Microbiol Biotechnol 70:564–572

Natasha Y, Lin YW, Gao YG, Zhao X, Russell BS, Lei L, Miner KD, Robinson H, Yi Lu Y (2009) Rational design of a structural and functional nitric oxide reductase. Nature 462:24–31

Nieto C, Tresguerres FE, Sanchez N, Vicente M, Diaz R (1990) Cloning vectors, derived from a naturally occurring plasmid of *Pseudomonas savastanoi*, specifically tailored for genetic manipulations in *Pseudomonas*. Gene 87:145–149

OECD (1992) Safety consideration for biotechnology. OECD, Paris

OECD (1994) Biotechnology for a clean environment prevention, detection, remediation. OECD, Paris

Okinaka Y, Yang CH, Perna NT, Keen NT (2002) Microarray profiling of *Erwinia chrysanthemi* 3937 genes that are regulated during plant infection. Mol Plant Microbe Interact 15:619–629

Paitan Y, Biran I, Shechter N, Biran D, Rishpon J, Ron EZ (2004) Monitoring aromatic hydrocarbons by whole cell electrochemical biosensor. Anal Biochem 335:175–183

Pan A, Tie F, Duau Z, Yang M, Wang Z, Ku L, Chen Z, Ru B (1994) A-domain of human metallothionein I-A can bind to metals in transgenic tobacco plants. Mol Gen Genet 242:666–674

Parikh MR, Matsumura I (2005) Site-saturation mutagenesis is more efficient than DNA shuffling for the directed evolution of β-fucosidase from β-galactosidase. J Mol Biol 352:621–628

Patel J, Zhang Q, Michael R, McKay L, Vincent R, Xu Z (2010) Genetic engineering of *Caulobacter crescentus* for removal of cadmium from water. Appl Biochem Biotechnol 160:232–243

Patricia AS, Jonna MC (2009) Horizontal gene transfer in metal and radionuclide contaminated soils. Methods Mol Biol 532:455–472

Paul D, Pandey G, Jain RK (2005) Suicidal genetically engineered microorganisms for bioremediation: need and perspectives. Bioessays 27:563–573

Perales-Vela HV, Penã-Castro JM, Canîzares-Villanueva RO (2006) Heavy metal detoxification in eukaryotic microalgae. Chemosphere 64:1–10

Perez-Rama M, Alonso JA, Lopez CH, Vaamonde ET (2002) Cadmium removal by living cells of the marine microalga *Tetraselmis suecica*. Bioresour Technol 84:265–270

Pieper DH, Reineke W (2000) Engineering bacteria for bioremediation. Curr Opin Biotechnol 11:262–270

Qin L, Wu YJ (2009) A fluorescent, genetically engineered microorganism that degrades organophosphates and commits suicide when required. Appl Microbiol Biotechnol 82:749–756

Qin J, Rosen BP, Zhang Y, Wang GJ, Franke S, Rensing C (2006) Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S adenosylmethionine methyltransferase. Proc Natl Acad Sci USA 103:2075–2080

Radice F, Orlandi V, Massa V, Cavalca L, Demarta A, Wood TK, Barbieri P (2006) Genotypic characterization and phylogenetic relations of *Pseudomonas* sp. OX1. Curr Microbiol 52:395–399

Rao SRV, Rubin HE, Alexander M (1982) Kinetics and extension of mineralization of organic chemicals at trace levels in fresh water and sewage. Appl Environ Microbiol 43:1139–1150

Reetz MT, Carballeira JD (2007) Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes. Nat Protoc 2(4):891

Reetz MT, Bocola M, Carballeira JD, Zha DX, Vogel A (2005) Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test. Angew Chem Int Ed 44:4192–4196

Ripp S, Nivens DE, Ahn Y, Werner C, Jarrel J, Easter JP, Cox CD, Burlage RS, Sayler GS (2000) Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. Environ Sci Technol 34: 846–853

Rojo F, Pieper DH, Engesser KH, Knackmuss HJ, Timmis KN (1987) Assemblage of *ortho* cleavage route for simultaneous degradation of chloro- and methylaromatics. Science 238:1395–1398

Ronchel MC, Ramos JL (2001) Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. Appl Environ Microbiol 67:2649–2656

Ruiz NO, Daniell H (2009) Genetic engineering to enhance mercury phytoremediation. Curr Opin Biotechnol 20:1–7

Ryan AH, Peter MC, Gary SM, Gary SL (2000) Level of detail in ecological risk assessments. Mar Pollut Bull 40:471–477

Ryu DDY, Nam DH (2000) Recent progress in biomolecular engineering. Biotechnol Prog 16:2–16

Sakaki T, Shinkyo R, Takita T, Ohta M, Inouye K (2002) Biodegradation of polychlorinated dibenzo-p-dioxins by recombinant yeast expressing rat CYP1A subfamily. Arch Biochem Biophys 401:91–98

Saleem M, Brim H, Hussain S, Arshad M, Leigh MB, Ziaul-hassan (2008) Perspectives on microbial cell surface display in bioremediation. Biotechnol Adv 26:151–161

Samuelson P, Wernerus H, Svedberg M, Stahl S (2000) Staphylococcal surface display of metal-binding polyhistidyl peptides. Appl Environ Microbiol 66: 1243–1248

Sandrine SM, Stephan C, Patrick C, Catherine LP, Doan TL, Gilles P (2003) Enhanced toxic metal accumulation in engineered bacterial cells expressing *arabidopsis thaliana* phytochelatin synthase. Appl Environ Biotechnol 69:490–494

Sato SI, Nam JW, Kasuga K, Nojiri H, Yamane H, Omori T (1997) Identification and characterization of genes encoding carbazole 1,9-dioxygenase in *Pseudomonas* sp. Strain CA10. J Bacteriol 197: 4850–4858

Sauge-Merle S, Cuine S, Carrier P, Lecomte-Pradines C, Luu DT, Peltier G (2003) Enhanced toxic metal accumulation in engineered bacterial cells expressing *Arabidopsis thaliana* phytochelatin synthase. Appl Environ Microbiol 69:490–494

Sayler GS, Cox CD, Burlage RS, Ripp S, Nivens DE, Werner C, Ahn Y, Matrubutham U (1999) Field release of a genetically engineered microorganism for polycyclic aromatic hydrocarbon bioremediation process monitoring and control. In: Fass R, Flashner Y, Reuveny S (eds) Novel approaches for bioremediation of organic pollution. Kluwer Academic/Plenum Publishers, New York, pp 242–254

Schanstra JP, Kingma J, Janssen DB (1996) Specificity and kinetics of haloalkane dehalogenase. J Biol Chem 271:14747–14753

Schue M, Dover LG, Besra GS, Parkhill J, Brown NL (2009) Sequence and analysis of a plasmid encoded mercury resistance operon from *Mycobacterium marinum* identify MerH, a new mercuric ion transporter. J Bacteriol 19:439–444

Schweizer HP (2001) Vectors to express foreign genes and techniques to monitor gene expression in Pseudomonads. Curr Opin Biotechnol 12(5):439–445

Seung HK, Singh S, Kim JY, Lee W, Mulchandani A, Chen W (2007) Bacteria metabolically engineered for enhanced phytochelatin production and cadmium accumulation. Appl Environ Biotechnol 73:6317–6320

Shinkyo R, Sakaki T, Takita T, Ohta M, Inouye K (2003) Generation of 2,3,7,8-TCDD metabolizing enzyme by modifying rat CYP1A1 through site directed mutagenesis. Biochem Biophys Res Commun 308:511–517

Shintani M, Habe H, Tsuda M, Omori T, Yamane H, Nojiri H (2005) Recipient range of IncP-7 conjugative plasmid pCAR2 from *Pseudomonas putida* HS01 is broader than from other *Pseudomonas* strains. Biotechnol Lett 27:1847–1853

Simon N (2006) Recent advancements in the bioremediation of polychlorinated dibenzo-*p*-dioxins. Basic Biotechnol eJ 2:160–164

Singh JS, Abhilash PC, Singh HB, Singh RP, Singh DP (2011) Genetically engineered bacteria: an emerging tool for environmental remediation and future perspectives. Gene 480(1–2):1–9, 36851

Smith E, van Elsas JD, van Veen JA (1992) Risks associated with the application of genetically modified microorganisms in terrestrial ecosystems. FEMS Microbiol Lett 88:263–278

Smutok O, Broda D, Smutok H, Dmytruk K (2011) Chromate-reducing activity of *Hansenula polymorpha* recombinant cells over-producing flavocytochrome *b*2. Chemosphere 83(4):449–454

Sousa C, Kotrba P, Ruml T, Cebolla A, deLorenzo V (1998) Metalloadsorption by *Escherichia coli* cells displaying yeast and mammalian metallothioneins anchored to the outer membrane protein LamB. J Bacteriol 180:2280–2284

Sriprang R, Hayashi M, Ono H, Takagi M, Hirata K, Murooka Y (2003) Enhanced accumulation of Cd2+ by a *Mesorhizobium* sp. transformed with a gene from *Arabidopsis thaliana* coding for phytochelatin synthase. Appl Environ Microbiol 69:179–796

Stemmer WPC (1994) Rapid evolution of a protein *in vitro* by DNA shuffling. Nature 370:389–391

Suenaga H, Watanabe T, Sato M, Ngadiman, Furukawa K (2002) Alteration of regiospecificity in biphenyl dioxygenase by active-site engineering. J Bacteriol 184:3682

Takayama K, Suye S, Kuroda K, Ueda M, Kitaguchi T, Tsuchiyama K, Fukuda T, Chen W, Mulchandani A (2006) Surface display of organophosphorus hydrolase on *Saccharomyces cerevisiae*. Biotechnol Prog 22:939–943

Tang J, Liu L, Hu S, Chen Y, Chen J (2009) Improved degradation of organophosphate dichlorvos by *Trichoderma atroviride* transformants generated by restriction enzyme-mediated integration (REMI). Bioresour Technol 100:480–483

Thomas CM (2000) Paradigms of plasmid organization. Mol Microbiol 37:485–491

Time Magazine (1975) Environment: oil-eating bug. Time; Sep. 22, 1975

Timmis KN, Pieper DH (1999) Bacteria designed for bioremediation. Trends Biotechnol 17:201–204

Torres B, Garcia J, Diaz E (2003) Plasmids as tools for containment. In: Plasmid biology. ASM, Washington, DC, pp 589–601

Ueda M, Tanaka A (2000a) Cell surface engineering of yeast: construction of arming yeast with biocatalyst. J Biosci Bioeng 90:125–136

Ueda M, Tanaka A (2000b) Genetic immobilization of proteins on the yeast cell surface. Biotechnol Adv 18:121–140

van Dillewijn P, Wittich RM, Caballero A, Ramos JL (2008) Type II Hydride Transferases from different microorganisms yield nitrite and diarylamines from polynitroaromatic compounds. Appl Environ Microbiol 74:6820–6823

Vardar G, Wood TK (2005) Alpha-subunit positions methionine 180 and glutamate 214 of *Pseudomonas stutzeri* OX1 toluene-o-xylene monooxygenase influence catalysis. J Bacteriol 187:1511–1514

Wackett LP (1998) Directed evolution of new enzymes and pathways for environmental biocatalysis. Ann N Y Acad Sci 864:142

Wang CL, Yang DC, Wabi M (2004) Directed molecular evolution by somatic hypermutation. Protein Eng Des Sel 17:659–664

Webster LR, McKenzie GH, Moriarty HT (2002) Organophosphate-based pesticides and genetic damage implicated in bladder cancer. Cancer Genet Cytogenet 133:112–117

Wojciech D, Andrzej B (1995) Monitoring environmental pollution in Poland by chemical analysis of Scots pine (*Pinus sylvestris* L.) needles. Environ Pollut 87:87–104

Wood TK (2008) Molecular approaches in bioremediation. Curr Opin Biotechnol 19:572–578

Wu CH, Thomas KW, Mulchandani A, Chen W (2005) Engineering plant-microbe symbiosis for rhizoremediation of heavy metals. Appl Environ Biotechnol 72:1129–1134

Wu G, Kang H, Zhang X, Shao H, Chu L, Ruan C (2010) A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities. J Hazard Mater 14:1–8

Yoshida N, Ikeda R, Okuno T (2006) Identification and characterization of heavy metal-resistant unicellular alga isolated from soil and its potential for phytoremediation. Bioresour Technol 97: 1843–1849

Yu Q, Matheickal JT, Latten J (1998) Heavy metal adsorption properties of marine algae *Durvillaea potatorum.* Ecklonia radiata and Laminaria japonica. Chinese J Chem Eng 6:68–72

Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WPC, del Cardayre SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646

Ligninolytic Enzymes in Environmental Management

12

K.K. Sharma, Deepti Singh, Sapna, Bijender Singh, and Ramesh Chander Kuhad

Abstract

By producing lignocellulose-degrading enzymes, saprotrophic litter-decomposing Basidiomycetes can significantly contribute to the turnover of soil organic matter. The production of lignin- and polysaccharide-degrading enzymes helps in converting the waste litter into value-added compost. White-rot fungi (WRF) have tremendous potential for biodegradation of a variety of industrial pollutants. The capability of WRF for biodegradation of xenobiotics and recalcitrant pollutants has generated a considerable research interest in this area of environmental biotechnology. The broad spectrum for biodegradation of pollutants is due to the extracellular and nonspecific nature of the enzyme system of fungi, comprising mainly of lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase, and laccase along with other ancillary enzymes. Differential biodegradation capabilities of WRF are mainly due to physiological differences among them, difference in their genetic makeup, and variable pattern and expression of complex lignin-modifying enzymes (LMEs). The activities of the LMEs can be increased by the addition of different low-molecular-mass mediators, mostly secreted by white-rot fungi themselves.

Keywords

Lignin • Laccase • White-rot fungi • Lignin-modifying enzymes • Biodegradation • Bioremediation

Introduction

Fungi are an important and diverse component of soil microbial communities. They provide essential ecosystem functions, such as decomposing organic matter, nutrient cycling, and in the case of mycorrhizal species, also nutrient transfer to plants. In forest ecosystems they are largely

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responsible for breakdown of the abundant large biopolymers cellulose, hemicellulose, lignin, and chitin (Dighton et al. 2005; Kellner and Vandenbol 2010). Recent report suggests the importance of both ascomycetes, as well as Basidiomycetes, in key biogeochemical cycles (Kellner and Vandenbol 2010).

In terrestrial environments, Basidiomycetes are one of the most ecologically significant groups of fungi involved in the breakdown of litter components. They constitute a major fraction of the living biomass responsible for efficient degradation of many recalcitrant organic compounds in soil litter and the humic layer (Dix and Webster 1995; Steffen et al. (2007a, b). An efficient group of litter-degrading organisms are litter-decomposing Basidiomycetes, which produce a wide variety of oxidoreductases and hydrolytic enzymes and are also able to degrade lignin, the most recalcitrant litter component (Steffen et al. 2000). In contrast, Benner et al. (1986), in a study of lignocellulose degradation by microbial samples from two freshwater and two marine habitats, stated that bacteria rather than fungi were the predominant degraders of lignocellulose in aquatic habitat.

Basidiomycetes also have tremendous potential for biodegradation of a variety of industrial pollutants. The broad spectrum for biodegradation of pollutants is due to the extracellular and nonspecific nature of the enzyme system of white-rot fungi (WRF), comprising mainly of lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and laccase along with other accessory enzymes (Table 12.1). The biodegradation capabilities of WRF for different pollutants are variable, mainly due to physiological differences among them and variable pattern and expression of complex lignin-modifying enzymes (LMEs) in the presence of chemically different compounds (Asgher et al. 2008).

Extracellular hydrolases and oxidoreductases are involved in the breakdown of lignocellulose and are produced by many known bacteria, actinomycetes, and ligninolytic fungi. Lignocellulytic enzymes and their biotechnological application have already been discussed in earlier papers, but there is still an ongoing interest, especially in their occurrence and environmental significance. Cellulases, in particular the complex consisting of endoglucanase, cellobiohydrolase, and beta-glucosidase, hydrolyze the long chains of cellulose, resulting in the liberation of cellobiose and finally glucose. Hemicelluloses, such as endo-1,4-β-xylanase or mannanase, are involved in the breakdown of different heterogeneous polysaccharide chains such as xylans and mannans.

Lignin, polysaccharides, and nitrogenous compounds contribute in the formation of humus (Varadachari and Ghosh 1984; Fustec et al. 1989; Inbar et al. 1989). The chemical pathway from organic matter to humus involves complex degradative and condensation reactions. According to Varadachari and Ghosh (1984), lignin is first degraded by extracellular enzymes to smaller units, which are then absorbed into microbial cells where they are partly converted to phenols and quinones. Thereafter, the substances are discharged together with oxidizing enzymes into the environment, where they get polymerized by a free-radical mechanism. Composting is a dynamic process carried out by a rapid succession of mixed microbial consortia including bacteria, actinomycetes, and fungi (Tuomela et al. 2000; Kellner and Vandenbol 2010).

A wide range of bacteria have been isolated from different compost environments, including species of *Pseudomonas, Klebsiella,* and *Bacillus,* e.g., *B. subtilis*, *B. licheniformis,* and *B. circulans* (Nakasaki et al. 1985; Strom 1985a, b; Falcon et al. 1987). Actinomycetes appear during the thermophilic phase as well as the maturation phase of composting and can occasionally become so numerous that they are visible on the surface of the compost. The genera of the thermophilic actinomycetes isolated from compost include *Nocardia*, *Streptomyces*, *Thermoactinomyces*, and *Micromonospora* (Waksman et al. 1939; Strom 1985a).

Lignin Degradation

Lignin-degrading Basidiomycetes, collectively referred to as white-rot fungi, are common inhabitants of forest litter and fallen trees. These are

Table 12.1 Ligninolytic enzymes produced by white-rot fungi

Enzyme	EC no.	Reaction catalyzed	Applications	References
Laccase	1.10.3.2	Phenol oxidation	Spore resistance; rhizomorph formation; pathogenesis; fruit bodies formation; pigments synthesis; lignin degradation	Yaropolov et al. (1994), Mayer and Staples (2002), Claus (2004), Minussi et al. (2007), and Maciel et al. (2010)
Lignin peroxidase	1.11.1.14	Phenol polymerization	Biodegradation of lignin defense of fungi against pathogens	Gold and Alic, (1993), Haglund, (1999), Piontek et al. (2001), Erden et al. (2009), Score et al. (1997), Trejo-Hernandez et al. (2001), and Maciel et al. (2010)
Manganese peroxidase	1.11.1.13	Phenol oxidation; oxidize Mn^{2+} to Mn^{3+}	Degradation of lignin interspecific fungal interactions	Hofrichter (2002), Score et al. (1997), Trejo-Hernandez et al. (2001), and Maciel et al. (2010)
Aryl-alcohol oxidase	1.1.3.7	H_2O_2 production		Martínez et al. (2009)
Glyoxal oxidase	1.2.3.5	H_2O_2 production		Martínez et al. (2009)
Manganese independent peroxidase	1.11.1.7	Activity on aromatic substrates		Wyatt and Broda (1995) and Ruiz-Dueñas et al. (2009)
Versatile peroxidase	1.11.1.16	Oxidizes Mn^{2+}; high redox potential aromatic compounds	Able to efficiently oxidize phenolic compounds and dyes that are the substrates of generic peroxidases and related peroxidases	Ruiz-Dueñas et al. (2009)
Cellobiose dehydrogenase	1.1.99.18	Lignin degradation; unite the hydrolytic and oxidative systems; dispose manganese (MnII) for MnP through precipitate reduction from manganese oxide (MnO_2)		Henriksson et al. (2000a, b), Kersten and Cullen (2007), and Carvalho et al. (2009)

the only microbes that have been convincingly shown to efficiently depolymerize, degrade, and mineralize all components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin. As such, white-rot fungi play an important role in the carbon cycle (Kersten and Cullen 2007).

From the chemical point of view, lignin is a heterogeneous, optically inactive polymer consisting of phenylpropanoid interunits, which are linked by several covalent bonds (e.g., aryl-ether, aryl-aryl, carbon-carbon bonds) (Hofrichter 2002). The polymer arises from laccase- and/or peroxidase-initiated polymerization of phenolic precursors via the radical coupling of their corresponding phenoxy radicals. It is synthesized by higher plants, reaching levels of 20–30% of the dry weight of woody tissue. Because of the bond types and their heterogeneity, lignin cannot be cleaved by hydrolytic enzymes as most other natural polymers. Therefore, lignin is degraded with the help of different nonspecific oxidoreductases which specifically attack the aromatic moieties, preferably phenolic structures. The most widely studied enzymes in this group are laccase, LiP, MnP, and several other peroxidases such as VP (Sharma and Kuhad 2008).

Lignolytic Enzymes and Their Occurrence

Extracellular oxidative enzymes involved in lignin depolymerization include an array of oxidases and peroxidases. These enzymes are responsible for generating highly reactive and nonspecific free radicals that affect lignin degradation. The nonspecific nature and extraordinary oxidation potential of the peroxidases have attracted considerable interest in the development of several bio-processes.

Laccase

Laccase (benzenediol, oxygen oxidoreductases, EC1.10.3.2) is one of the few lignin-degrading enzymes that have been extensively studied since the eighteenth century. Laccases are majorly reported from eukaryotes, e.g., fungi, plants, and insects (Mayer and Staples 2002). However, some evidences for its existence in prokaryotes, with typical features of multicopper oxidase enzyme family, have also been reported (Alexandre and Zulin 2000). The first bacterial laccase was detected in the plant root-associated bacterium, *Azospirillum lipoferum* (Givaudan et al. 1993), where it was shown to be involved in melanin formation (Faure et al. 1994). A typical laccase containing six putative copper binding sites was discovered in marine bacterium *Marinomonas mediterranea*, but no functional role was assigned to this enzyme (Solano et al. 1997; Sanchez-Amat et al. 2001). In insects, laccases have been suggested to be active in cuticle sclerotization (Dittmer et al. 2004). Two isoforms of *laccase 2* gene have been found to catalyze larval, pupal, and adult cuticle tanning in *Tribolium castaneum* (Arakane et al. 2005), and a novel laccase has been isolated and characterized from a bovine rumen metagenome library that neither exhibited any sequence similarity to known laccases nor contained hitherto identified functional laccase motifs (Beloqui et al. 2006).

Recently, Sharma and Kuhad (2009), has reported 22 COGs from Archaea, bacteria, and eukaryotes (http://img.jgi.doe.gov. and http://www.ncbi.nlm.nih.gov/cog). Genome-specific best hit resulted in very exhaustive genomic information of diverse multicopper oxidases. Laccase (CotA) from *B. subtilis* 168 and *B. pumilus* SAFR-032 was found to share a common clade and close ancestry with multicopper oxidase from *Pyrobaculum aerophilum*, an Archaea. Moreover, *P. aerophilum* was also found to be evolutionary related to *E. coli* APEC O1 (laccase) and *Yersinia pestis* KIM (hypothetical protein). Well-known laccases from *T. versicolor* were found to be closely related to *Neurospora crassa* OR74A, *C. neoformans* var. *neoformans* JEC21, and *Drosophila melanogaster*, a common fruit fly. Multicopper oxidases from different yeast, i.e., FET3_Yeast, *Pichia stipitis* CBS6054 (FET3.1), and *Saccharomyces cerevisiae* (FET5), share a common phylogenetic position. An unusual evolutionary history was also established between pathogenic Proteobacteria, i.e., *Burkholderia mallei* and *Burkholderia pseudomallei*, and an archaeal species, i.e., *Haloarcula marismortui* ATCC 43049 and *Natronomonas pharaonis* DSM2160 (Sharma

and Kuhad 2009). Moreover, laccase has been extensively examined since the mid-1970s, and a number of reviews have appeared on the subject (Malkin et al. 1969; Malmstrom et al. 1975; Holwerda et al. 1976; Mayer and Harel 1979; Reinhammar 1984; Thurston 1994; Eriksson 2000; Xu 2005; Morozova et al. 2007; Sharma et al. 2007; Sharma and Kuhad 2008).

Lignin Peroxidase

Lignin depolymerization is catalyzed by extracellular peroxidases of white-rot Basidiomycetes such as *Phanerochaete chrysosporium* (Tien and Kirk 1983). Lignin peroxidase (LiP) was first discovered based on the H_2O_2-dependent C_α–C_β cleavage of lignin model compounds and subsequently shown to catalyze the partial depolymerization of methylated lignin in vitro (Glenn et al. 1983; Tien and Kirk 1983; Gold et al. 1984; Tien and Kirk 1984). Due to their high redox potentials and their enlarged substrate range in the presence of specific mediators, LiPs have great potential for application in various industrial processes (Paice et al. 1995). LiP, being a heme-containing glycoprotein with an unusually low pH optimum (Glumoff et al. 1990), is able to catalyze the oxidation of a variety of compounds with reduction potentials exceeding 1.4 V (vs. normal hydrogen electrode) (Steenken 1998). Contrary to other heme peroxidases, ferric LiP is first oxidized by H_2O_2 to compound I, a two-electron-oxidized intermediate, which is then reduced by one substrate molecule to the second intermediate, compound II. Further reduction back to the resting enzyme can be accomplished either by the same substrate molecule or a second one.

Manganese Peroxidase

Manganese peroxidase (MnP) is considered to be the most common lignin-modifying peroxidase produced by almost all wood-colonizing Basidiomycetes (Tien and Kirk 1983; Martínez et al. 2005). Multiple forms of this glycosylated heme protein with molecular weights normally at 40–50 kDa are secreted by ligninolytic fungi into their microenvironment. There, MnP preferentially oxidizes manganese (II) ions (Mn^{2+}), always present in wood and soils, into highly reactive Mn^{3+}, which is stabilized by fungal chelators such as oxalic acid. Chelated Mn^{3+} in turn acts as low-molecular-weight, diffusible redox mediator that attacks phenolic lignin structures resulting in the formation of instable free radicals that tend to disintegrate spontaneously (Kuwahara et al. 1984; Hofrichter 2002).

Versatile Peroxidase

Versatile peroxidase (VP) has been recently described as a new family of ligninolytic peroxidases, together with lignin peroxidase (LiP) and manganese peroxidase (MnP), both reported for *P. chrysosporium* for the first time. The complete genome of this model fungus has been recently sequenced revealing two families of LiP and MnP genes together with a "hybrid peroxidase" gene. Till date, VP has been reported from the genera *Pleurotus, Bjerkandera, Lepista, Trametes,* and *Panus* (Honda et al. 2006; Rodakiewicz-Nowak et al. 2006). The most noteworthy aspect of VP is that it combines the substrate specificity characteristics of the three other fungal peroxidase families. In this way, it is able to oxidize a variety of (high and low redox potential) substrates including Mn^{2+}, phenolic, and non-phenolic lignin dimers, α-keto-γ-thiomethylbutyric acid (KTBA), veratryl alcohol, dimethoxybenzenes, different types of dyes, substituted phenols, and hydroquinones (Ruiz-Dueñas et al. 2009).

Glyoxal Oxidases

An important component of the ligninolytic system of *P. chrysosporium is* the H_2O_2 that is required as oxidant in the peroxidative reactions. Glyoxal oxidases have been proposed to play a role in this regard (Kirk and Farrell 1987). The temporal correlation of glyoxal oxidase, peroxidase, and oxidase substrate appearances in cultures suggests a close physiological connection between these components (Kersten and Kirk 1987; Kersten 1990). It is a glycoprotein of 68 kDa with two isozymic forms (pI 4.7 and 4.9). The active site of the enzyme has not been characterized, but Cu^{2+} appears to be important in maintaining activity of purified enzyme. Glyoxal oxidase is produced in cultures when *P. chrysosporium* is grown on glucose or xylose, the major sugar components of lignocellulosics. The physiological substrates for glyoxal oxidase, however, are not these

growth-carbon compounds, but their intermediates. A number of simple aldehyde, α-hydroxycarbonyl, and α-dicarbonyl compounds are the known substrates (Cullen and Kersten 1996).

The reversible inactivation of glyoxal oxidase is a property perhaps of considerable physiological significance (Kersten 1990; Kurek and Kersten 1995). Glyoxal oxidase becomes inactive during enzyme turnover in the absence of a coupled peroxidase system. The oxidase is reactivated, however, by lignin peroxidase and non-phenolic peroxidase substrates. Conversely, phenolics prevent the activation by lignin peroxidase. This suggests that glyoxal oxidase has a regulatory mechanism in the presence of peroxidases, their substrates, and their products (e.g., phenolics resulting from ligninolysis). Notably, lignin will also activate glyoxal oxidase in the coupled reaction with LiP (Cullen and Kersten 1996). Cellobiose oxidase (Ayers et al. 1978) and cellobiose: quinone oxidoreductase (CBQase) (Westermark and Eriksson, 1974) may be involved in both lignin and cellulose degradation. Limited proteolysis of cellobiose oxidase indicates that CBQase is probably a breakdown product (Henriksson et al. 1991; Wood and Wood 1992). Cellobiose oxidase has two domains, one containing a flavin and the other containing a heme group. The flavin-containing domain binds cellulose and is functionally similar to CBQase. A role proposed for these oxidoreductases is to prevent repolymerization of phenoxy radicals produced by peroxidases and laccases during lignin oxidation (Eriksson and Goldman 1993; Cullen and Kersten 1996). Moreover the peroxide-generating enzyme, i.e., pyranose oxidase (glucose-2-oxidase), which is intracellular in liquid culture condition of *P. chrysosporium*, plays an additional important role in wood decay (Daniel et al. 1994).

Environmental Significance

Bioremediation technology utilizes the metabolic potential of microorganisms to clean up the environment (Watanabe 2001). Lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and versatile peroxidase (VPs) are the major LMEs of WRF involved in lignin and xenobiotic degradation by white-rot fungi (Pointing 2001) (Table 12.1). Accessory enzymes such as H_2O_2-forming glyoxal oxidase, aryl-alcohol oxidase, oxalate producing oxalate decarboxylase (ODC), NAD-dependent formate dehydrogenase (FDH), and P450 monooxygenase have also been isolated from many white-rot fungal strains (Doddapaneni et al. 2005; Aguiar et al. 2006). Lignin peroxidases (LiPs) are capable of mineralizing a variety of recalcitrant aromatic compounds (Srivastava et al. 2005). Due to nonspecific nature, lignin-oxidizing enzyme is capable of mineralizing a wide variety of toxic xenobiotics and recalcitrant substrates. In recent years, a lot of work has been done on the development and optimization of bioremediation processes using WRF, with emphasis on the study of their enzyme systems involved in biodegradation of industrial waste (Thurston 1994; Eriksson 2000; Baldrian 2006; Sharma and Kuhad 2008) (Table 12.1).

Bioremediation of Industrial Pollutant

Bioremediation process employs microorganisms or plants to remove the contaminating organic compounds by metabolizing them to carbon dioxide and biomass (Alexander 1994). The purpose of bioremediation is to degrade pollutants to undetectable concentrations or to concentrations that are below the limits established by regulatory agencies. Bioremediation has been used to degrade contaminants in soils, ground water, wastewater, sludges, industrial waste, and gases (Alexander 1994).

Biodegradation of Synthetic Dye

Large amounts of structurally diverse dyestuffs are used for textile dyeing as well as other applications. Based on the chemical structure of the chromophoric group, dyes are classified as azo dyes, anthraquinone dyes, phthalocyanine dyes, etc. (Kuhad et al. 2004). Different dyes and pigments are extensively used in the textile, paper, plastic, cosmetics, pharmaceutical, and food industries (Levin et al. 2005). The involvement of LMEs in the dye decolorization process has been confirmed in several

independent studies using purified cell-free enzymes (Table 12.2). LiP of *P. chrysosporium* has been shown to decolorize azo, triphenylmethane, and heterocyclic dyes in the presence of veratryl alcohol and H_2O_2 (Cripps et al. 1990; Ollikka et al. 1993). Selected Basidiomycetes have been observed to decolorize PolyR-478 (Vasdev and Kuhad 1994) and various triphenylmethane dyes (Vasdev et al. 1995). Laccase can act on chromophoric compounds such as Remazol Brilliant Blue R or triphenylmethane dyes and suggests a potential application in bleaching or decolorization industrial processes (Vasdev et al. 1995).

Further, interest in the biodegradation of synthetic dyes has primarily been prompted by concern over their possible toxicity and carcinogenicity (Maas and Chaudhari 2005; Revankar and Lele 2007). White-rot fungi are better dye degraders than prokaryotes due to their extracellular nonspecific LME system capable of degrading a wide range of dyes (Christian et al. 2005). Most of the earlier dye decolorization studies were based mainly on *P. chrysosporium* and *T. versicolor* (Toh et al. 2003). However, other white-rot fungi including *Phellinus gilvus*, *Pleurotus sajor-caju*, *Pycnoporus sanguineus* (Balan and Monteiro 2001), *Dichomitus squalens*, Irpex flavus, *Daedalea flavida*, *Polyporus sanguineus* (Chander et al. 2004; Eichlerová et al. 2006; Chander and Arora 2007), *Funalia trogii* ATCC200800 (Ozsoy et al. 2005), *Ischnoderma resinosum* (Eichlerová et al. 2006), and *Ganoderma* sp. WR-1 (Revankar and Lele 2007) have been demonstrated to have higher dye decolorization rates than *P. chrysosporium* and *Trametes versicolor* (Table 12.2).

Biodegradation of Polycyclic Aromatic Hydrocarbon

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants that occur in soils, sediments, airborne particles, freshwater, and marine environments (Bumpus 1989). PAHs are nonpolar, neutral, organic molecules that comprise two or more fused benzene rings arranged in various configurations, including linear, angular, and clustered alignments (Collins et al. 1996).

There have been several reports to use bioremediation of PAHs. Eukaryotic microorganisms, such as fungi, cannot use PAHs as a sole carbon source for growth but usually co-metabolize the PAH to dead-end metabolites. In contrast, bacteria can completely degrade many PAHs and use them as the sole carbon and energy source for growth (Sutherland 1992). At present, many microorganisms are known to metabolize the lower-molecular-weight PAHs, but these PAHs tend not to be highly carcinogenic. Less is known about the potential for biodegradation of higher-molecular-weight PAHs, which tend to be more carcinogenic. A microorganism's ability to degrade PAHs is dependent on the bioavailability of the compound (Vandertol-Vanier 2000).

White-rot fungi can completely mineralize some polycyclic aromatic hydrocarbons (PAHs), indicating that complete oxidation of PAHs occurs. However, there are few examples of in vitro oxidation of PAHs by culture supernatants and purified enzymes. The oxidation of anthracene and pyrene by lignin and manganese peroxidases from *P. chrysosporium* and oxidation of many PAHs by the laccases of *T. versicolor* have been reported (Bumpus 1989; Collins et al. 1996). Pickard et al. (1999) have shown that previously uncharacterized fungal strains could metabolize selected PAHs in vivo. *C. gallica* was one of the strains studied and was found to degrade several PAHs. Anthracene concentration decreased by up to 90%; pyrene, up to 20%; and phenanthrene, up to 40% (Vandertol-Vanier 2000) (Table 12.2).

TNT and Other Explosives

The explosives TNT, HMX, and RDX are integral components of many armaments. Degradation of TNT was studied by Donnelly et al. in 1997, using four different strains of white-rot fungi *P. chrysosporium*, *Phanerochaete sordida*, *Phlebia brevispara*, and *Cyathus stercoreus* in liquid medium (Donnelly et al. 1997). They found that within 21 days of incubation, all fungi were able to reduce the TNT concentration (from 90 mg/L) in the liquid medium to below detection limits. *P. sordida* showed a relatively high growth rate and the fastest rate of TNT degradation. White-rot fungi were also found to degrade monoamino-dinitrotoluenes, the major chemical metabolites in the initial transformation of TNT. The studies

Table 12.2 Applications of ligninolytic enzyme producing organism in treatment of environmental pollution

Application	Organism	References
Decolorization of dyes	*Aspergillus* (recombinant)	Soares et al. (2001a)
	Aspergillus (recombinant)	Soares et al. (2001b)
	A. niger	Soares et al. (2002)
	Cerrena unicolor	Michniewicz et al. (2003)
	Coriolopsis gallica	Reyes et al. (1999)
	C. rigida	Gómez et al. (2005)
	Funalia trogii	Ünyayar et al. (2005)
	Ganoderma sp. WR-1	Revankar and Lele (2007)
	Irpex lacteus	Kasinath et al. (2003)
	Laetiporus sulphureus and *Coriolus versicolor*	Mazmanci et al. (2009)
	Myceliophthora thermophila, *Polyporus pinsitus*, *Trametes versicolor*	Claus et al. (2002)
	Pleurotus eryngii, *Pycnoporus cinnabarinus*, *T. versicolor*	Camarero et al. (2004)
	Pleurotus ostreatus	Hou et al. (2004), Palmieri et al. (2005)
	P. cinnabarinus	McCarthy et al. (1999) and Schliephake et al. (2000)
	Sclerotium rolfsii, *Trametes hirsute*	Campos et al. (2001)
	Streptomyces cyaneus	Arias et al. (2003)
	Stereum ostrea	Viswanath et al. (2008)
	S. maltophilia AAP56	Dube et al. (2008)
	T. hirsuta	Abadulla et al. (2000), Domínguez et al. (2005), Moldes et al. (2003), Rodríguez Couto et al. (2004b, 2005, 2006), Rodríguez Couto and Sanromán (2006), Couto and Toca-Herrera (2006a, b), Minussi et al. (2007), Nyanhongo et al. (2002), Levin et al. (2005), and Maceiras et al. (2001)
	T. modesta	Rodríguez Couto et al. (2002)
	T. trogii	Kulys et al. (2003) and Peralta-Zamora et al. (2003)
	T. versicolor	Maceiras et al. (2001), Lorenzo et al. (2002), and Blánquez et al. (2004)
	T. villosa	Potin et al. (2004), Saito et al. (2004), Tavares et al. (2004), Zille et al. (2003), Knutson and Ragauskas (2004), Yamanaka et al. (2008), Ciullini et al. (2008), and Yang et al. (2009)
	Trametes sp. *strain SQ01*	Pickard et al. (1999)
	Strain I-4 of the family *Chaetomiaceae*	Vandertol-Vanier et al. (2002)
Degradation of xenobiotics	*Cladosporium sphaerospermum*	Cho et al. (2002)
	Coprinus cinereus, *Myceliophthora thermophila*, *P. pinsitus*, *Rhizoctonia solani*	Itoh et al. (2000)
	C. gallica	Okazaki et al. (2002), Nicotra et al. (2004), and Casa et al. (2003)
	Coriolus hirsutus	Zavarzina et al. (2004)
	Coriolus versicolor	Eggen (1999) and Hublik and Schinner (2000)
	Myceliophthora thermophila, *Trametes pubescens*	Keum and Li (2004)
	Panus tigrinus	Mougin et al. (2002)

(continued)

Table 12.2 (continued)

Application	Organism	References
	P. ostreatus	Lante et al. (2000) and Carunchio et al. (2001)
	P. ostreatus, *T. versicolor*	Moeder et al. (2004)
	P. cinnabarinus	Niku-Paavola and Viikari (2000)
	Pyricularia oryzae	Böhmer et al. (1988) and Tanaka et al. (2001)
	Rhus vernicifera	Tanaka et al. (2003)
	T. hirsute	Collins et al. (1996) and Johannes et al. (1998)
	Trametes sp.	Majcherczyk et al. (1998) and Johannes and Majcherczyk (2000)
	T. versicolor	Majcherczyk and Johannes (2000) and Castro et al. (2003)
	T. villosa	Dodor et al. (2004), Fabbrini et al. (2001), Fukuda et al. (2001), Kang et al. (2002), Cantarella et al. (2003), and Jung et al. (2003)
	Trichophyton sp. LKY-7	Steffen et al. (2007a, b), Cabana et al. (2007), and Cambria et al. (2008)
	Stropharia rugosoannulata	Calvo et al. (1998)
	Stropharia coronilla	Murugesan (2003)
	Coriolopsis polyzona	D'Annibale et al. (1999)
	Rigidoporus lignosus	D'Annibale et al. (2000)
	Gliocladium virens	D'Annibale et al. (2004)
Effluent treatment	*Lentinula edodes*	Tsioulpas et al. (2002), Aggelis et al. (2003), and Jaouani et al. (2005)
	P. tigrinus	Durante et al. (2004)
	Pleurotus spp.	Jolivalt et al. (2000)
	Pycnoporus coccineus	Edwards et al. (2002)
	R. vernicifera	Lucas et al. (2003)
	Trametes sp. strain AH28-2	Pedroza et al. (2007)
	T. versicolor	Cordi et al. (2007)
	Lentinula edodes	Cordi et al. (2007)
	Botrytis cinerea	Ellouze et al. (2008), Bourbonnais et al. (1997), and Call and Mücke (1997)
	Trametes trogii	Archibald et al. (1997)
	Lentinus tigrinus	Crestini and Argyropoulos (1998)
	Fomes fomentarius, *Ganoderma callosum*, *Lentinus edodes*, *Merulius tremellosus*, *Phlebia radiata*, *P. ostreatus*, *T. versicolor*	Kandioller and Christov (2001)
	C. versicolor	Cordi et al. (2007)
	T. versicolor	Cordi et al. (2007)
Biopulping	*T. versicolor*	Paice et al. (1995) and Cordi et al. (2007)
	Peniophora sp., *Pycnoporus sanguineus*, *T. hirsuta*, *T. versicolor*	Oudia et al. (2008)
	T. versicolor, T. villosa	Balakshin et al. (2001)
	Lentinula edodes	Camarero et al. (2004)
	Botrytis cinerea	Georis et al. (2003)
	C. versicolor	Archibald et al. (1997)
	P. eryngii, P. cinnabarinus, T. versicolor	Bastos and Magan (2009)
	P. cinnabarinus	D'Souza-Ticlo et al. (2009)
Biobleaching	*T. versicolor*	Molina-Guijarro et al. (2009)
	T. versicolor	Punnapayak et al. (2007) and Zhao et al. (2010)

established that white-rot fungi are capable of metabolizing and detoxifying TNT under aerobic conditions in a non-ligninolytic liquid medium. The degradation of TNT by white-rot fungi is a two-step process: the first step was to be degraded to OHADNT and ADNT, and the second step was to DANT (Aken et al. 1999). As reported by Axtell et al. (2000), the strains of *P. chrysosporium* and *P. ostreatus* adapted to grow on high concentrations of TNT thus were able to cause extensive degradation of TNT, HMX, and RDX.

Bioremediation of Contaminated Sites

Many pesticides, xenobiotics, coal substances, and industrial products derived from polycyclic, aromatic, halogenated hydrocarbons, and other organic compounds are hazardous environment pollutants. Using oxidoreductases to detoxify and remove them is attracting active research efforts. Laccase and peroxidase have been used to transform (often in the presence of redox mediators) various xenobiotics, polycyclic aromatic hydrocarbons, and other pollutants found in industrial waste and contaminated soil or water (Xu 2005).

Contrary to most of the research on bioremediation using bacterial strains, fungal bioremediation has attracted in the past few years. White-rot fungi have potential to withstand toxic levels of most organopollutants. Five main genera of white-rot fungi have shown potential for bioremediation, *viz*., *Phanerochaete, Trametes, Bjerkandera, Pleurotus,* and *Cyathus* (Table 12.2). These fungi cannot use lignin as a sole source of energy, however, instead require substrates such as cellulose or other carbon sources. Thus, carbon sources such as corncobs, straw, and sawdust can be easily used to enhance degradation rates by these organisms at polluted sites. Also, the branching, filamentous mode of fungal growth allows for more efficient colonization and exploration of contaminated soil. The main mechanism of biodegradation employed by this group of fungi, however, is the use of lignin degradation system of enzymes. The enzymes LiP, MnP, and laccase involved in lignin degradation are highly nonspecific with regard to their substrate range; this is not surprising considering their mode of action via the generation of radicals (Reddy and Mathew 2001; Kapoor et al. 2005).

Degradation of Medical Waste

Exposure to alkyl-substituted polynuclear aromatic hydrocarbons, stilbenes, genistein, methoxychlor and endocrine-disrupting chemicals (EDC), nonylphenol (NP) and bisphenol A (BPA), and the personal care product ingredient triclosan (TCS) (Asgher et al. 2008) has been associated with a variety of reproductive responses in fish (Kiparisis et al. 2003). Degradation of genistein by *Phanerochaete sordida* YK-624 and detection of the activities of ligninolytic enzymes, MnP, and laccase during treatment show the involvement of WRF extracellular lignolytic system in disappearance of genistein (Tamagawa et al. 2005). MnP, laccase, and the laccase-HBT systems of WRF are also effective in removing the estrogenic activities of bisphenol A (BPA), nonylphenol (NP), 17b-estradiol (E2), and ethinylestradiol (EE2) with production of high-molecular-weight oligomeric metabolites (Asgher et al. 2008; Lee et al. 2005). Further, removal of NP and BPA is associated with the production of laccase by *T. versicolor* and *Bjerkandera* sp. BOL13 (Soares et al. 2005, 2006). The enhanced biocatalytic elimination of nonylphenol (NP), bisphenol A (BPA), and triclosan (TCS) by *Coriolopsis polyzona* by the addition of ABTS (Cabana et al. 2007) also suggested the involvement of laccase-mediator system.

The ligninolytic enzymes of white-rot fungi catalyze the degradation of pollutants by using a nonspecific free-radical mechanism. When an electron is added or removed from the ground state of a chemical, it becomes highly reactive, allowing it to give or take electrons from other chemicals. This provides the basis for the nonspecificity of the enzymes and their ability to degrade xenobiotics, chemicals that have never been encountered in nature (Pointing 2001).

Biodegradation of Rubber Industry Waste

Recycling of spent rubber material is problematic due to the vulcanization, which creates strong sulfur bonds between the rubber molecules (Liu et al. 2000). Different processes for desulfurization of rubber material and to facilitate the reuse of waste rubber have been developed, including biotechnological processes (Bredberg et al. 2002). Microbial devulcanization is a promising way

to increase the recycling of rubber materials. However, several microorganisms tested for devulcanization are sensitive to rubber additives (Christiansson et al. 2000; Asgher et al. 2008). Most of the common rubber additives are aromatic compounds and can be effectively removed by LMEs of WRF. *Resinicium bicolor* is the most effective fungus for detoxification of rubber material, especially the ground waste tire rubber (Bredberg et al. 2002). Treatment of aromatic rubber additives with *R. bicolor* enhances the growth of *Thiobacillus ferrooxidans* bacterium as well as desulfurization compared to the untreated rubber (Asgher et al. 2008).

Control of Pitch in Paper Pulp Manufacturing

Wood extractives cause production and environmental problems in pulp and paper manufacturing. The lipophilic compounds, which form the so-called wood resin, are the most problematic, and they include free fatty acids, resin acids, waxes, fatty alcohols, sterols, sterol esters, glycerides, ketones, and other oxidized compounds. During wood pulping and refining of paper pulp, the lipophilic extractives in the parenchyma cells and softwood resin canals is released, forming colloidal pitch. These colloidal particles can coalesce into larger droplets that deposit in pulp or machinery forming "pitch deposits" or remain suspended in the process waters. Pitch deposition has a detrimental environmental impact when released into wastewaters (Gutiérrez et al. 2001).

The ability to colonize lignified plant material is a characteristic of wood decay fungi, which include white-rot, brown-rot, soft-rot, and sapstain species. The fungi that cause white rot and brown rot are Basidiomycetes and are characterized by their ability to degrade lignin and cellulose, respectively, resulting in white, i.e., cellulose or brown-colored, i.e., lignin-enriched decayed substrates. The typical sapstain fungi, also called "blue-staining fungi," colonize wood vessels and rays (as well as softwood resin canals) penetrating through the cell-wall pits. The growth of sapstain fungi is supported by easily degradable extractives and causes discoloration and minimal weight loss. Wood discoloration is caused by the presence of melanin that has a role in the protection of fungal hyphae against harmful radiation. Because most lipophilic compounds involved in the formation of pitch deposits are concentrated in wood rays and resin canals, the sapstain fungi were the first candidates for the biological control of pitch during wood pulping. Wood-rotting Basidiomycetes have also been investigated for biotechnological application in paper pulp manufacturing. Brown-rot fungi are of little applied interest because they degrade cellulose, the most valuable wood constituent for industrial utilization. Biopulping, in combination with chemical and mechanical treatments, represents an attractive alternative to reduce the consumption of pulping chemicals and energy. White-rot fungi and their enzymes are also of biotechnological interest for pulp bleaching. The advantages of WRF in the degradation of lipophilic extractives have also being realized. The main purpose of biobleaching is to reduce the consumption of the chlorinated reagents traditionally used to bleach pulp, which have a detrimental impact in the water environment (Gutiérrez et al. 2001).

Enzymatic Pulp Bleaching

New environmentally benign, elemental chlorine-free (ECF), and totally chlorine-free (TCF) bleaching technologies are necessary for minimizing the hemicellulose content in dissolving pulp, adjusting the brightness at a high level and improving, simultaneously, the quality of the effluent in terms of toxicity and absorbable organic halogen (AOX). Biological methods of pulp prebleaching using xylanases (Taneja et al. 2002) provide the possibility of selectively removing up to 20% of xylan from pulp and saving up to 25% of chlorine-containing bleaching chemicals. Alternatively, pulp can be bleached with white-rot fungi and their ligninolytic enzymes, enabling chemical savings to be achieved and a chlorine-free bleaching process.

Bjerkandera sp. strain BOS55, *Polyporus ciliatus*, *Stereum hirsutum*, *Phlebia radiata,* and *Lentinus tigrinus* have been found to be efficient biobleachers (Akhtar et al. 1992). Kirk and Yang

(1979) were the first to attempt to bleach pulp with *P. chrysosporium* and some other white-rot fungi. This could lower the kappa number of unbleached softwood kraft pulp up to 75%, leading to reduced requirement for chlorine during the subsequent chemical bleaching. *T. versicolor* could markedly increase the brightness of hardwood kraft pulp. The fungal treatment was carried out in agitated, aerated cultures for 5 days. The kappa number was decreased from 12 to 8, and the brightness increased by 34–48%. *P. cinnabarinus* was found to produce laccase and also its own laccase redox mediator, 3-hydroxy anthranilic acid (3-HAA) (Eggert et al. 1996). The presence of laccase is essential for lignin degradation by *P. cinnabarinus* and that in its absence pulp bleaching is greatly reduced. The biobleaching of kraft with laccase mediator continues to receive strong interest, in part due to the discovery of new mediators for laccase. A number of mediators have recently been used for the use of laccase enzyme in biobleaching, e.g., ABTS 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (Bourbonnais and Paice 1996), HBT, *N*-acetyl-*N*-phenylhydroxylamine (NHA) and violuric acid (VA) (Chakar and Ragauskas 2004). HBT oxidation leads to the discovery of a new class of mediators with NOH as the reactive species (R-NO). Kraft pulp treatment with laccase and ABTS was found to effectively demethylate and delignify hardwood kraft pulp when the mediator ABTS is present (Bourbonnais and Paice 1996).

Laccase, like other phenol-oxidizing enzymes, such as peroxidases (Huttermann et al. 1980; Haemmerli et al. 1986; Kern and Kirk 1987), preferentially polymerizes lignin by coupling of the phenoxy radicals produced by the oxidation of lignin phenolic groups. When laccase is used alone, the only reaction that can be observed on kraft lignin is polymerization. The fact that ABTS prevents polymerization of kraft lignin by laccase cannot be explained only by inhibition or reduction of the lignin phenoxy radicals produced by laccase, because when ABTS was added after lignin polymerization by laccase, the lignin was effectively depolymerized. It seems likely that ABTS functions as a diffusible electron carrier, because laccase is a large molecule and therefore cannot enter the secondary wall to contact the lignin substrate directly.

Conclusion

The ligninolytic enzymes of white-rot fungi catalyze the degradation of pollutants by using a nonspecific free-radical mechanism. The enzymes LiP, MnP, laccase, and other ancillary enzymes involved in lignin degradation are highly nonspecific with regard to their substrate range. This is not surprising considering their mode of action via the generation of radicals. This provides the basis for the nonspecificity of the enzymes and their ability to degrade xenobiotics and other industrial waste that have never been encountered as a natural substrate and are deleterious to ecosystem. Lignolytic enzyme system holds potential for cleaning the degraded and contaminated sites, using combinatorial, holistic, and ecofriendly approaches.

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References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gübitz G (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 66:3357–3362

Aggelis G, Iconomou D, Christouc M, Bokas D, Kotzailias S, Christou G et al (2003) Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process. Water Res 37:3897–3904

Aguiar A, de Souza-Cruz PB, Ferraz A (2006) Oxalic acid, Fe^{3+}-reduction activity and oxidative enzymes detected in culture extracts recovered from *Pinus taeda* wood chips biotreated by *Ceriporiopsis subvermispora*. Enzyme Microbial Technol 38(7):873–878

Aken BV, Hofrichter M, Scheibner K, Hatakka AI, Naveau H, Agathos SN (1999) Transformation and mineralization of 2,4,6-trinitrotoluene (TNT) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata*. Biodegradation 10:83–91

Akhtar M, Attridge MC, Blanchette RA, Myers GC, Wall MB, Sykes MS, Koning Jr JW, Burgess RR, Wegner TH, Kirk T (1992) Biotechnology in pulp and paper

industry. In: Proceedings of the 5th international conference on biotechnology in the pulp and paper industry, University publishers Ltd, Tokyo, pp 3–8
Alexander M (1994) Biodegradation and bioremediation. Academic, San Diego, pp 177–195
Alexandre G, Zulin IB (2000) Laccases are widespread in bacteria. Trends Biotechnol 18:41–42
Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, Kramer KJ (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc Natl Acad Sci USA 102:11337–11342
Archibald FS, Bourbonnais R, Jurasek L, Paice MG, Reid ID (1997) Kraft pulp bleaching and delignification by *Trametes versicolor*. J Biotechnol 53:215–236
Arias ME, Arenas M, Rodríguez J, Soliveri J, Ball AS, Hernández M (2003) Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl Environ Microbiol 69:1953–1958
Asgher M, Bhatti HN, Ashraf M, Legge RL (2008) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19(6):771–783
Axtell C, Johnston CG, Bumpus JA (2000) Bioremediation of soil contaminated with explosives at the Naval Weapons Station Yorktown. Soil Sediment Contam Int J 9:537–548
Ayers AR, Ayers SB, Eriksson KE (1978) Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*. Eur J Biochem 90:171–181
Balan DSL, Monteiro RTR (2001) Decolorization of textile indigo dye by ligninolytic fungi. J Biotechnol 89(2–3):141–145
Balakshin M, Chen C-L, Gratzl JS, Kirkman AG, Jakob H (2001) Biobleaching of pulp with dioxygen in laccase-mediator system-effect of variables on the reaction kinetics. J Mol Catal B Enzyme 16:205–215
Baldrian P (2006) Fungal laccases: occurrence and properties. FEMS Microbiol Rev 30:215–242
Bastos AC, Magan N (2009) *Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions. Int J Biodeterior Biodegrad 63:389–394
Beloqui A, Pita M, Polaina J et al (2006) Novel polyphenol oxidase mined from metagenome expression library of bovine rumen: biochemical properties structural analysis and phylogenetic relationship. J Biol Chem 281:22933–22942
Benner R, Moran MA, Hodson RE (1986) Biogeochemical cycling of lignocellulosic carbon in marine and freshwater ecosystems: relative contribution of prokaryotes and eukaryotes. Limnol Oceanogr 31:89–100
Blánquez P, Casas N, Font X, Gabarrell M, Sarrá M, Caminal G (2004) Mechanism of textile metal dye biotransformation by *Trametes versicolor*. Water Res 38:2166–2172
Böhmer S, Messner K, Srebotnik E (1988) Oxidation of phenanthrene by a fungal laccase in the presence of 1-hydroxybenzotriazole and unsaturated lipids. Biochem Biophys Res Commun 244:233–238
Bourbonnais R, Paice MG (1996) Enzymic delignification of kraft pulp using laccase and a mediator. Tappi J 79:199–204
Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63:4627–4632
Bredberg K, Andersson BE, Landfors E, Holst O (2002) Microbial detoxification of waste rubber material by wood-rotting fungi. Bioresour Technol 83:221–224
Bumpus J (1989) Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 55:154–158
Cabana H, Jiwan JL, Rozenberg R, Elisashvili V, Penninckx M, Agathos SN, Jones JP (2007) Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using preparation from the white rot fungus *Coriolopsis polyzona*. Chemosphere 67:770–778
Call HP, Mücke I (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator systems (LignozymR process). J Biotechnol 53:163–202
Calvo AM, Copa-Patiño JL, Alonso O, González AE (1998) Studies of the production and characterization of laccase activity in the basidiomycete *Coriolopsis gallica*, an efficient decolorizer of alkaline effluents. Arch Microbiol 171:31–36
Camarero S, Garcia O, Vidal T, Colom J, del Rio JC, Gutierrez A et al (2004) Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. Enzyme Microb Technol 35:113–120
Cambria MT, Minniti Z, Librando V, Cambria A (2008) Degradation of polycyclic aromatic hydrocarbons by *Rigidoporus lignosus* and its laccase in the presence of redox mediators. Appl Biochem Biotechnol 149:1–8
Campos R, Kandelbauer A, Robra KH, Cavaco-Paulo A, Gübitz GM (2001) Indigo degradation with purified laccases from *Trametes hirsute* and *Sclerotium rolfsii*. J Biotechnol 89:131–139
Cantarella G, Galli C, Gentili P (2003) Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase/mediator systems. Catalytic or stoichiometric procedures. J Mol Catal B Enzyme 22:135–144
Carunchio F, Crescenzi C, Girelli AM, Messina A, Tarola AM (2001) Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides. Talanta 55:189–200
Carvalho W, Canilha L, Ferraz A, Milagres A (2009) Uma visão sobre a estrutura, composição e biodegradação da madeira. Química Nova 32(8):1–5
Casa R, D'Annibale A, Pieruccetti F, Stazi SR, Giovannozzi SG, LoCascio B (2003) Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. Chemosphere 50:959–966

Castro AIRP, Evtuguin DV, Xavier AMB (2003) Degradation of biphenyl lignin model compounds by laccase of *Trametes* versicolor in the presence of 1 hydroxybenzotriazole and heteropolyanion [SiW11VO40]5−. J Mol Catal B Enzyme 22:13–20

Chakar FS, Ragauskas AJ (2004) Biobleaching chemistry of laccase-mediator systems on high-lignin-content kraft pulps. Can J Chem Rev Can Chim 82(2):344–352

Chander M, Arora DS (2007) Evaluation of some white-rot fungi for their potential to decolorize industrial dyes. Dyes Pigments 72:192–198

Chander M, Arora DS, Bath HK (2004) Biodecolourisation of some industrial dyes by white-rot fungi. J Ind Microbiol Biotechnol 31:94–97

Cho SJ, Park SJ, Lim JS, Rhee YH, Shin KS (2002) Oxidation of polycyclic aromatic hydrocarbons by laccase of *Coriolus hirsutus*. Biotechnol Lett 24:1337–1340

Christiansson M, Stenberg B, Holst O (2000) Toxic additives - a problem for microbial waste rubber desulfurisation. Res Environ Biotechnol 3:11–21

Ciullini I, Tilli S, Scozzafava A, Fabrizio B (2008) Fungal laccase, cellobiose dehydrogenase, and chemical mediators: combined actions for the decolorization of different classes of textile dyes. Bioresour Technol 99:7003–7010

Claus H (2004) Laccases: structure, reactions, distribution. Micron 35(1–2):93–96

Claus H, Faber G, König H (2002) Redox-mediated decolorization of synthetic dyes by fungal laccases. Appl Microbiol Biotechnol 59:672–678

Collins PJ, Kotterman MJJ, Field JA, Dobson ADW (1996) Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*. Appl Environ Microbiol 62:4563–4567

Cordi L, Minnussi RC, Freire RS, Duran N (2007) Fungal laccase: copper induction, semi-purification, immobilization, phenolic effluent treatment and electrochemical measurement. Afr J Biotechnol 6:1255–1259

Couto RS, Toca-Herrera JL (2006a) Laccases in the textile industry. Biotechnol Mol Biol Rev 1:115–120

Couto SR, Toca-Herrera JL (2006b) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24:500–513

Crestini C, Argyropoulos DS (1998) The early oxidative biodegradation steps of residual kraft lignin models with laccase. Bioorg Med Chem 6:2161–2169

Cripps C, Bumpus JA, Aust SD (1990) Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. Appl Environ Microbiol 56(4):11–14

Cullen D, Kersten PJ (1996) Enzymology and molecular biology of lignin degradation. In: Bramble R, Marzluf G (eds) The Mycota III. Springer, Berlin/Heidelberg/New York, pp 297–314

Daniel G, Volc J, Kubatova E (1994) Pyranose oxidase, a major source of H_2O_2 during wood degradation by Phanerochaete chrysosporium, Trametes versicolor, and Oudemansiella mucida. Appl Environ Microbiol 60:2524–2532

D'Annibale A, Stazi SR, Vinciguerra V, Di Mattia E, Giovannozzi SG (1999) Characterization of immobilized laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. Process Biochem 34:697–706

D'Annibale A, Stazi SR, Vinciguerra V, Giovannozzi SG (2000) Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. J Biotechnol 77:265–273

D'Annibale A, Ricci M, Quaratino D, Federic F, Fenice M (2004) *Panus tigrinus* efficiently removes phenols, color and organic load from olive-mill wastewater. Res Microbiol 155:596–603

D'Souza-Ticlo D, Sharma D, Raghukumar C (2009) A thermostable metal-tolerant laccase with bioremediation potential from a marine-derived fungus. Marine Biotechnol 11:725–737

Dighton J, White JF, Oudemans P (2005) The fungal community: its organization and role in the ecosystem. Taylor & Francis, Boca Raton, 936 p

Dittmer NT, Suderman RJ, Jiang H, Zhu YC, Gorman MJ, Kramer KJ, Kanost MR (2004) Characterization of cDNA encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. Insect Biochem Mol Biol 34:29–41

Dix NJ, Webster J (1995) Fungal ecology. Chapman & Hall, London

Doddapaneni H, Chakraborty R, Yadav JS (2005) Genome-wide structural and evolutionary analysis of the P450 monooxygenase genes (P450ome) in the white rot fungus *Phanerochaete chrysosporium*: evidence for gene duplications and extensive gene clustering. BMC Genom 6:92–116

Dodor DE, Hwang HM, Ekunwe SIN (2004) Oxidation of anthracene and benzo[a] pyrene by immobilized laccase from *Trametes versicolor*. Enzyme Microb Technol 35:210–217

Domínguez A, Rodríguez Couto S, Sanromán MA (2005) Dye decolourization by *Trametes hirsuta* immobilised into alginate beads. World J Ind Microbiol Biotechnol 21:405–409

Donnelly KC, Chen JC, Huebner HJ, Brown KW, Autenrieth RL, Bonner JS (1997) Utility of four strains of white-rot fungi for the detoxification of 2,4,6-trinitrotoluene in liquid culture. Environ Toxicol Chem 16:1105–1110

Dube E, Shareck F, Hurtubise Y, Daneault C, Beauregard M (2008) Homologous cloning, expression, and characterization of a laccase from *Streptomyces coelicolor* and enzymatic decolourization of an indigo dye. Appl Microbiol Biotechnol 79:597–603

Durante D, Casadio R, Martelli L, Tasco G, Portaccio M, De Luca P et al (2004) Isothermal and non-isothermal bioreactors in the detoxification of waste waters polluted by aromatic compounds by means of immobilised laccase from *Rhus vernicifera*. J Mol Catal B Enzyme 27:191–206

Edwards W, Leukes WD, Bezuidenhout J (2002) Ultrafiltration of petrochemical industrial wastewater using immobilised manganese peroxidase and laccase:

application in the defouling of polysulphone membranes. J Desalin 149:275–278

Eggen T (1999) Application of fungal substrate from commercial mushroom production *Pleuorotus ostreatus* for bioremediation of creosote contaminated soil. Int Biodeterior Biodegrad 44:117–126

Eggert C, Temp U, Eriksson KE (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol 62: 1151–1158

Eichlerova I, Homolka L, Nerud F (2006) Synthetic dye decolorization capacity of white rot fungus *Dichomitus squalens*. Bioresour Technol 97:2153–2159

Ellouze M, Aloui F, Sayadi S (2008) Detoxification of Tunisian landfill leachates by selected fungi. J Hazard Mater 150:642–648

Erden E, Ucar CM, Gezer T, Pazarlioglu NK (2009) Screening for ligninolytic enzymes from autochthonous fungi and applications for decolorization of Remazole Marine Blue. Braz J Microbiol 40(2):346–353

Eriksson JE, Goldman RD (1993) Protein phosphatase inhibitors alter cytoskeletal structure and cellular morphology. Adv Protein Phosphatases 7:335–357

Eriksson K-EL (2000) Lignocellulose, lignin, ligninases. In: Encyclopedia microbiol, vol III, II edn. Academic

Fabbrini M, Galli C, Gentili P, Macchitella D (2001) An oxidation of alcohols by oxygen with the enzyme laccase and mediation by TEMPO. Tetrahedron Lett 42:7551–7553

Falcon MA, Corominas E, Perez ML, Perestelo F (1987) Aerobic bacterial populations and environmental factors involved in the composting of agricultural and forest wastes of the Canary Islands. Biol Wastes 20:89–99

Faure D, Bouillant ML, Bally R (1994) Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. Appl Environ Microbiol 60:3413–3415

Fukuda T, Uchida H, Takashima Y, Uwajima T, Kawabata T, Suzuki M (2001) Degradation of bisphenol a by purified laccase from *Trametes villosa*. Biochem Biophys Res Commun 284:704–706

Fustec E, Chauvet E, Gas G (1989) Lignin degradation and humus formation in alluvial soils and sediments. Appl Environ Microbiol 55(4):922–926

Georis J, Lomascolo A, Camarero S, Dorgeo V, Herpoel I, Asther M (2003) *Pycnoporus cinnabarinus* laccases: an interesting tool for food or non-food applications. Meded Fac Landbouwkd Toegep Biol Wet 68: 263–266

Givaudan A, Effose A, Faure D, Potier P, Bouillant M-L, Bally R (1993) Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. FEMS Microbiol Lett 108:205–210

Glenn JK, Morgan MA, Mayfield MB, Kuwahara M, Gold MH (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 114(3): 1077–1083

Glumoff T, Harvey PJ, Molinari S, Goble M, Frank G, Palmer JM, Smit JDG, Leisola MSA (1990) Lignin peroxidase from *Phanerochaete chrysosporium*. Molecular and kinetic characterization of isozymes. Eur J Biochem 187(3):515–520

Gold MH, Enoki A, Morgan MA, Mayfield MB, Tanaka H (1984) Degradation of the γ-carboxyl-containing diarylpropane lignin model compound 3-(4,-Ethoxy-3,-Methoxyphenyl)-2-(4″-Methoxyphenyl) Propionic acid by the basidiomycete phanerochaete chrysosporium. Appl Environ Microbiol 47(4):597–600

Gold MH, Alic M (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol Rev 57(3):605–622

Gómez J, Pazos M, Rodríguez Couto S, Sanromán MA (2005) Chestnut shell and barley bran as potential substrates for laccase production by *Coriolopsis rigida* under solid-state conditions. J Food Eng 68:315–319

Gutiérrez A, del Río JC, Martínez MJ, Martínez AT (2001) The biotechnological control of pitch in paper pulp manufacturing. Trends Biotechnol 19(9):340–348

Haemmerli SD, Leisola MSA, Fiechter A (1986) Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium*. FEMS Microbiol Lett 35:33–36

Haglund C (1999) Biodegradation of xenobiotic compounds by the white-rot fungus *Trametes trogii*. Molecular Biotechnology Programme, Uppsala University School of Engineering, Uppsala, 30 p

Henriksson G, Pettersson G, Johansson G, Ruiz A, Uzcategui E (1991) Cellobiose oxidase from *Phanerochaete chrysosporium* can be cleaved by papain into two domains. Eur J Biochem 196:101–106

Henriksson G, Johansson G, Pettersson G (2000a) A critical review of cellobiose dehydrogenases. J Biotechnol 78(2):93–113

Henriksson G, Zhang L, Li J, Ljungquist P, Reitberger T, Pettersson G, Johansson G (2000b) Is cellobiose dehydrogenase from *Phanerochaete chrysosporium* a lignin degrading enzyme? Biochim Biophys Acta (BBA) Protein Struct Mol Enzymol 1480(1–2):83–91

Hofrichter M (2002) Review: lignin conversion by manganese peroxidase (MnP). Enzyme Microb Technol 30(4):454–466

Holwerda RA, Wherland S, Gray HB (1976) Electron transfer reactions of copper proteins. Annu Rev Biophys Bioeng 5:363

Honda Y, Watanabe T, Watanabe T (2006) Exclusive overexpression and structure-function analysis of a versatile peroxidase from white-rot fungus, *Pleurotus ostreatus*. Sustain Humanosphere 2:2–6

Hou H, Zhou J, Wang J, Du C, Yan B (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. Process Biochem 39:1415–1419

Hublik G, Schinner F (2000) Characterization and immobilization of the laccase from *Pleurotus ostreatus* and

its use for the continuous elimination of phenolic pollutants. Enzyme Microb Technol 27:330–336

Huttermann A, Herche C, Haars A (1980) Polymerization of water-insoluble lignins by *Fomes Annosus*. Holzforschung 34:64–66

Inbar Y, Chen Y, Hadar Y (1989) Solid-state Carbon-13 nuclear magnetic resonance and infrared spectroscopy of composted organic matter. Soil Sci Soc Am J 53:1695–1701

Itoh K, Fujita M, Kumano K, Suyama K, Yamamoto H (2000) Phenolic acids affect transformations of chlorophenols by a *Coriolus versicolor* laccase. Soil Biol Biochem 32:85–91

Jaouani A, Guillen F, Penninckx MJ, Martinez AT, Martinez MJ (2005) Role of *Pycnoporus coccineus* laccase in the degradation of aromatic compounds in olive oil mill wastewater. Enzyme Microb Technol 36:478–486

Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66:524–528

Johannes C, Majcherczyk A, Huttermann A (1998) Oxidation of acenaphthene and acenaphthylene by laccase of *Trametes versicolor* in a laccase-mediator system. J Biotechnol 61:151–156

Jolivalt C, Brenon S, Caminade E, Mougin C, Pontié M (2000) Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater. J Membr Sci 180:103–113

Jung H, Hyun K, Park Ch (2003) Production of laccase and bioremediation of pentachlorophenol by wood-degrading fungus *Trichophyton* sp. LKY-7 immobilized in Ca-alginate beads. Polpu Chongi Gisul 35:80–86

Kandioller G, Christov L (2001) Evaluation of the delignification and bleaching abilities of selected laccases with HBT on different pulps. In: Argyropoulos DS (ed) Oxidative delignification chemistry fundamentals and catalysis, vol 785, ACS symposium series. Oxford University Press, New York, pp 427–443

Kang KH, Dec J, Park H, Bollag JM (2002) Transformation of the fungicide cyprodinil by a laccase of *Trametes villosa* in the presence of phenolic mediators and humic acid. Water Res 36:4907–4915

Kapoor RK, Sharma KK, Kuhar S, Kuhad RC (2005) Diversity of lignin degrading microorganisms, lignolytic enzymes and their biotechnological applications. In Satyanarayana T, Johri BN (eds) Microbial diversity: current perspectives and potential applications. I. K. International Pvt. Ltd., New Delhi, pp 815–84

Kasinath A, Svobodová NK, Patel KC, Šašek V (2003) Decolorization of synthetic dyes by *Irpex lacteus* in liquid cultures and packed-bed bioreactor. Enzyme Microb Technol 32:167–173

Kellner H, Vandenbol M (2010) Fungi unearthed: transcripts encoding lignocellulolytic and chitinolytic. Enzym Forest Soil 5(6):e10971

Kern HW, Kirk TK (1987) Influence of molecular size and ligninase pretreatment on degradation of lignins by *Xanthomonas* sp. Appl Environ Microbiol 53: 2242–2246

Kersten PJ, Kirk TK (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J Bacteriol 169:2195–2201

Kersten PJ (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc Natl Acad Sci USA 87:2936–2940

Kersten P, Cullen D (2007) Extracellular oxidative systems of the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. Fungal Genet Biol 44(2):77–87

Keum YS, Li QX (2004) Fungal laccase-catalyzed degradation of hydroxyl polychlorinated biphenyls. Chemosphere 56:23–30

Kiparisis Y, Balch GC, Metcalfe TL, Metcalfe CD (2003) Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*). Environ Health Perspect 111:1158–1163

Kirk TK, Yang HH (1979) Partial delignification of unbleached kraft pulp with ligninolytic fungi. Biotechnol Lett 1:347–352

Kirk TK, Farrell RL (1987) Enzymatic combustion: the microbial degradation of lignin. Annu Rev Microbiol 41:465–505

Knutson K, Ragauskas A (2004) Laccase-mediator biobleaching applied to a direct yellow dyed paper. Biotechnol Prog 20:1893–1896

Kuhad RC, Kapoor M, Rustagi R (2004) Enhanced production of an alkaline pectinase from *Streptomyces* sp. RCK-SC by whole-cell immobilization and solid-state cultivation. World J Microbiol Biotechnol 20:257–263

Kulys J, Vidziunaite R, Schneider P (2003) Laccase-catalyzed oxidation of naphthol in the presence of soluble polymers. Enzyme Microb Technol 32:455–463

Kurek B, Kersten PJ (1995) Physiological regulation of glyoxal oxidase from *Phanerochaete chrysosporium* by peroxidase systems. Enzyme Microb Technol 17:751–756

Kuwahara M, Glenn JK, Morgan MA, Gold MH (1984) Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett 169: 247–250

Lante A, Crapisi A, Krastanov A, Spettoli P (2000) Biodegradation of phenols by laccase immobilised in a membrane reactor. Process Biochem 36:51–58

Lee Y, Yoon J, Gunten UV (2005) Kinetics of the oxidation of phenols and phenolic endocrine disruptors during water treatment with ferrate (Fe(VI)). Environ Sci Technol 39:8978–8984

Levin L, Forchiassin F, Viale A (2005) Ligninolytic enzyme production and dye decolorization by *Trametes trogii*: application of the Plackett–Burman experimental design to evaluate nutritional requirements. Process Biochem 40:1381–1387

Liu HS, Mead JL, Stacer RG (2000) Environmental effects of recycled rubber in light-fill applications. Rubber Chem Technol 73:551–564

Lorenzo M, Moldes D, Rodríguez Couto S, Sanromán A (2002) Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. Bioresour Technol 82:109–113

Lucas M, La D, Rubia T, Martinez J (2003) Oxidation of low molecular weight aromatic components of olive-mill wastewaters by a *Trametes versicolor* laccase. Polyphenols Actual 23:36–47

Maas R, Chaudhari S (2005) Adsorption and biological decolourization of azo dye Reactive Red 2 in semicontinuous anaerobic reactors. Process Biochem 40(2):699–705

Maceiras R, Rodríguez Couto S, Sanromán A (2001) Influence of several inducers on the synthesis of extracellular laccase and in vivo decolourisation of Poly R-478 by semi-solid-state cultures of *Trametes versicolor*. Acta Biotechnol 21:255–264

Maciel MJM, Silva AC, Ribeiro HCT (2010) Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: a review. Electron J Biotechnol 13(6):14–15

Majcherczyk A, Johannes C (2000) Radical mediated indirect oxidation of a PEG-coupled polycyclic aromatic hydrocarbon (PAH) model compound by fungal laccase. Biochim Biophys Acta 1474:157–162

Majcherczyk A, Johannes C, Hüttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enzyme Microb Technol 22:335–341

Malkin R, Malmstrom BG, Vanngard T (1969) The reversible removal of one specific copper (II) from fungal laccase. Eur J Biochem 7:253

Malmstrom BG, Andreason LE, Reinhammar R (1975) In: Boyer PD (ed) The enzymes, vol 12B, 3rd edn. Academic, New York, p 507

Martínez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Guillén F, Martínez MJ, Gutiérrez A, del Rio CJ (2005) Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 8:195–204

Martínez AT, Ruiz-Dueñas FJ, Martínez MJ, Del Rio JC, Gutiérrez A (2009) Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotechnol 20(3):348–357

Mayer AM, Harel E (1979) Polyphenol oxidases in plants. Phytochemistry 33:765–767

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60(6):551–565

Mazmanci MA, Ali U, Erkurt EA, Arkey NB, Bilen E, Ozyurt M (2009) Colour removal of textile dyes by culture extracts obtained from white rot fungi. Afr J Microbiol Res 3:585–589

Mccarthy JT, Levy VC, Lonergan GT, Fecondo JV (1999) Development of optimal conditions for the decolourisation of a range of industrial dyes using *Pycnoporus cinnabarinus* laccase. Hazard Ind Waste 31:489–498

Michniewicz A, Ledakowicz S, Jamroz T, Jarosz-Wilkolazka A, Leonowicz A (2003) Decolorization of aqueous solution of dyes by the laccase complex from *Cerrena unicolor*. Biotechnologia 4:194–203

Minussi RC, Miranda MA, Silva JA, Ferreira CV, Aoyama H, Marangoni S, Rotilio D, Pastore GM, Durán N (2007) Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hidroxybenzotriazole. Afr J Biotechnol 6(6):1248–1254

Moeder M, Martin C, Koeller G (2004) Degradation of hydroxylated compounds using laccase and horseradish peroxidase immobilized on microporous polypropylene hollow fiber membranes. J Membr Sci 245:183–190

Moldes D, Gallego PP, Rodríguez Couto S, Sanromán A (2003) Grape seeds: the best lignocellulosic waste to produce laccase by solid state cultures of *Trametes hirsuta*. Biotechnol Lett 25:491–495

Molina-Guijarro JM, Perez J, Munoz-Dorado J, Guillen F, Moy R, Hernandez M, Arias ME (2009) Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. Int Microbiol 12:13–21

Morozova OV, Shumakovich GP, Shleev SV, Yaropolov Ya I (2007) Laccase–mediator systems and their applications: a review. Appl Biochem Microbiol 43(5):523–535

Mougin C, Jolivalt C, Malosse C, Chaplain V, Sigoillot JC, Asther M (2002) Interference of soil contaminants with laccase activity during the transformation of complex mixtures of polycyclic aromatic hydrocarbons in liquid media. Polycycl Aromat Compd 22:673–688

Murugesan K (2003) Bioremediation of paper and pulp mill effluents. Indian J Exp Biol 41:1239–1248

Nakasaki K, Sasaki M, Shoda M, Kubota H (1985) Characteristic of mesophilic bacteria isolates isolated during thermophilic composting of sewage sludge. Appl Environ Microbiol 49:42–45

Nicotra S, Cramarossa MR, Mucci A, Pagnoni UM, Riva S, Forti L (2004) Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from *Myceliophthora thermophyla* and from *Trametes pubescens*. Tetrahedron 60:595–600

Niku-Paavola M-L, Viikari L (2000) Enzymatic oxidation of alkenes. J Mol Catal B Enzyme 10:435–444

Nyanhongo GS, Gomes J, Gübitz G, Zvauya R, Read JS, Steiner W (2002) Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresour Technol 84:259–263

Okazaki S-Y, Michizoe J, Goto M, Furusaki S, Wariishi H, Tanaka H (2002) Oxidation of bisphenol A catalyzed by laccase hosted in reversed micelles in organic media. Enzyme Microb Technol 31:227–232

Oudia A, Queiroz J, Simoes R (2008) The influence of operating parameters on the biodelignification of *Eucalyptus globules* Kraft pulps in a laccase-violuric acid system. Appl Biochem Biotechnol 149:149–3

Ozsoy S, Altunatmaz K, Horoz H, Kasikcle G, Alkan S, Bilat T (2005) The relationship between lameness, fertility and aflatoxin in a dairy cattle herd. Turk J Vet Anim Sci 29:981–986

Paice MG, Bourbonnais R, Reid ID (1995) Bleaching kraft pulps with oxidative enzymes and alkaline hydrogen peroxide. Tappi J 78:161–169

Palmieri G, Cennamo G, Sannia G (2005) Remazol brilliant blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. Enzyme Microb Technol 36:17–24

Pedroza AM, Mosqueda R, Alonso-Vante N, Rodriguez-Vazquez R (2007) Sequential treatment via *Trametes versicolor* and UV/TiO2/Ru(x)Se(y) to reduce contaminants in waste water resulting from the bleaching process during paper production. Chemosphere 67:793–801

Peralta-Zamora P, Pereira CM, Tiburtius ERL, Moraes SG, Rosa MA, Minussi RC (2003) Decolorization of reactive dyes by immobilized laccase. Appl Catal B Environ 42:131–144

Pickard MA, Roman R, Tinoco R, Vazquez-Duhalt R (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl Environ Microbiol 65:3805–3809

Piontek K, Smith AT, Blodig W (2001) Lignin peroxidase structure and function. Biochem Soc Trans 29(2): 111–116

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57(1–2):20–33

Potin O, Veignie E, Rafin C (2004) Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. FEMS Microbiol Ecol 51:71–78

Punnapayak H, Prasongsuk S, Messner K, Danmek K, Lotrakul P (2007) Polycyclic aromatic hydrocarbons (PAHs) degradation by laccase from a tropical white rot fungus *Ganoderma lucidum*. Afr J Biotechnol 8:5897–5900

Reddy CA, Mathew Z (2001) In: Gadd GM (ed) Bioremediation potential of white rot fungi. Cambridge University Press, Cambridge, p 250

Reinhammar B (1984) Laccase. In: Lontie R (ed) Copper proteins and copper enzymes, vol 3. CRC Press, Boca Raton, pp 1–35

Revankar MS, Lele SS (2007) Synthetic dye decolorization by white rot fungi, *Ganoderma* sp. WR-1. Bioresour Technol 98:775–780

Reyes P, Pickard MA, Vazquez-Duhalt R (1999) Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. Biotechnol Lett 21:875–880

Rodakiewicz-Nowak J, Jarosz-Wilkołazka A, Luterek J (2006) Catalytic activity of versatile peroxidase from *Bjerkandera fumosa* in aqueous solutions of water-miscible organic solvents. Appl Catal A Gen 308:56–61

Rodríguez Couto S, Sanromán MA (2006) Effect of two wastes from groundnut processing on laccase production and dye decolourization ability. J Food Eng 73:388–393

Rodríguez Couto S, Gundín M, Lorenzo M, Sanromán A (2002) Screening of supports for laccase production by *Trametes versicolor* in semisolid - state conditions. Determination of optimal operation conditions. Process Biochem 38:249–255

Rodríguez Couto S, Rosales E, Gundín M, Sanromán MA (2004) Exploitation of a waste from the brewing industry for laccase production by two *Trametes* sp. J Food Eng 64:423–428

Rodríguez Couto S, Sanromán MA, Gübitz GM (2005) Influence of redox mediators and metal ions on synthetic acid dye decolourization by crude laccase from *Trametes hirsuta*. Chemosphere 58:417–422

Rodríguez Couto S, López E, Sanromán MA (2006) Utilisation of grape seeds for laccase production in solid-state fermentors. J Food Eng 74:263–267

Ruiz-Dueñas FJ, Morales M, García E, Miki Y, Martínez MJ, Martínez AT (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 60(2):441–452

Saito T, Kato K, Yokogawa Y, Nishida M, Yamashita N (2004) Detoxification of bisphenol A and nonylphenol by purified extracellular laccase from a fungus isolated from soil. J Biosci Bioeng 98:64–66

Sanchez-Amat A, Lucas-Elio P, Fernandez E, Garcia-Borron JC, Solano F (2001) Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea*. Biochim Biophys Acta 1547:104–116

Schliephake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL (2000) Transformation and degradation of the diazodye Chicago Sky Blue by a purified laccase from Pycnoporus cinnabarinus. Enzyme Microb Technol 27:100–107

Score AJ, Palfreyman JW, White NA (1997) Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. Int Biodeter Biodegr 39(2–3):225–233

Sharma KK, Kuhad RC (2008) Laccase: enzyme revisited and functions redefined. Indian J Microbiol 48: 309–316

Sharma KK, Kuhad RC (2009) An evidence of laccase in archaea. Indian J Microbiol 49:142–150

Sharma P, Goel R, Capalash N (2007) Bacterial laccases. World J Microbiol Biotechnol 23:823–832

Soares GMB, Costa-Ferreira M, Pessoa de Amorim MT (2001a) Decolorization of an anthraquinone-type dye using a laccase formulation. Bioresour Technol 79:171–177

Soares GMB, Pessoa de Amorim MT, Costa-Ferreira M (2001b) Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. J Biotechnol 89:123–129

Soares GMB, Pessoa Amorim MT, Hrdina R, Costa-Ferreira M (2002) Studies on the biotransformation of novel disazo dyes by laccase. Process Biochem 37: 581–587

Soares A, Jonasson K, Terrazas E, Guieysse B, Mattiasson B (2005) The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol. Appl Microbiol Biotechnol 66:719–725

Soares A, Guieysse B, Mattiasson B (2006) Influence of agitation on the removal of nonylphenol by the white-rot fungi *Trametes versicolor* and *Bjerkandera* sp. BOL 13. Biotechnol Lett 28:139–143

Solano F, Garcia E, Perez D, Egea E, Sanchez-Amat A (1997) Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. Appl Environ Microbiol 63:3499–3506

Srivastava V, Negi AS, Kumar JK, Gupta MM, Khanuja SPS (2005) Plant-based anticancer molecules: a chemical and biological profile of some important leads. Bioorg Med Chem 13:5892–5908

Steenken S (1998) Lifetime, reduction potential and base-induced fragmentation of the veratryl alcohol radical cation in aqueous solution. Pulse radiolysis studies on a ligninase "Mediator". J Phys Chem A 102:7337–7342

Steffen KT, Hofrichter M, Hatakka A (2000) Mineralisation of 14C-labelled synthetic lignin and ligninolytic enzyme activities of litter decomposing basidiomycetous fungi. Appl Microbiol Biotechnol 54:819–825

Steffen KT, Schubert S, Tuomela M, Hattaka A, Hofrichter M (2007a) Enhancement of bioconversion of high-molecular mass polycyclic aromatic hydrocarbons in contaminated non-sterile soils by litter-decomposing fungi. Biodegradation 18:359–363

Steffen TK, Cajthaml T, Šnajdr J, Baldrian P (2007b) Differential degradation of oak (*Quercus petraea*) leaf litter by litter-decomposing basidiomycetes. Res Microbiol 158:447–455

Strom PF (1985a) Effect of temperature on bacterial species diversity in thermophilic solid-waste composting. Appl Environ Microbiol 50:899–905

Strom PF (1985b) Identification of thermophilic bacteria in solid- waste composting. Appl Environ Microbiol 50:907–913

Sutherland JB (1992) Detoxification of polycyclic aromatic hydrocarbons by fungi. J Ind Microbiol 9:53–62

Tamagawa Y, Hirai H, Kawai S, Nishida T (2005) Removal of estrogenic activity of endocrine-disrupting genistein by ligninolytic enzymes from white rot fungi. FEMS Microbiol Lett 244:93–98

Tanaka T, Tonosaki T, Nose M, Tomidokoro N, Kadomura N, Fujii T (2001) Treatment of model soils contaminated with phenolic endocrine-disrupting chemicals with lactase from *Trametes* sp. in a rotating reactor. J Biosci Bioeng 92:312–316

Tanaka T, Nose M, Endo A, Fujii T, Taniguchi M (2003) Treatment of nonylphenol with laccase in a rotating reactor. J Biosci Bioeng 96:541–546

Taneja K, Gupta S, Kuhad RC (2002) Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresour Technol 85(1):39–42

Tavares APM, Gamelas JAF, Gaspar AR, Evtuguin DV, Xavier AMRB (2004) A novel approach for the oxidative catalysis employing polyoxometalate–laccase system: application to the oxygen bleaching of kraft pulp. Catal Commun 5:485–489

Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26

Tien M, Kirk K (1983) Lignin-degrading enzyme from the hymenocete *Phanerochaete chrysosporium*. Science 221:661–663

Tien M, Kirk TK (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc Natl Acad Sci USA 81(8):2280–2284

Toh Y-C, Yen JJL, Obbard JP, Ting Y-P (2003) Decolourisation of azo dyes by white-rot fungi (WRF) isolated in Singapore. Enzym Microb Technol 33(5):569–575

Trejo-Hernandez MR, Lopez-Munguia A, Quintero Ramirez R (2001) Residual compost of *Agaricus bisporus* as a source of crude laccase for enzymatic oxidation of phenolic compounds. Process Biochem 36(7):635–639

Tsioulpas A, Dimou D, Iconomou D, Aggelis G (2002) Phenolic removal in olive oil mill wastewater by strains of *Pleurotus* spp. In respect to their phenol oxidase (laccase) activity. Bioresour Technol 84:251–257

Tuomela M, Vikman M, Hatakka A, Itavaara M (2000) Biodegradation of lignin in a compost environment: a review. Bioresour Technol 72:169–183

Ünyayar A, Mazmanci MA, Ataçağ H, Erkurt EA, Coral G (2005) A Drimaren Blue X3LR dye decolorizing enzyme from *Funalia trogii* one step isolation and identification. Enzyme Microb Technol 36:10–16

Vandertol-Vanier HA (2000) The role of laccase from C*oriolopsis gallica* in polycyclic aromatic hydrocarbon metabolism, PhD thesis Faculty of Graduate Studies and Research, Dept. of Biological Sciences, University of Alberta, Canada

Vandertol-Vanier HA, Vazquez-Duhalt R, Tinoco R, Pickard MA (2002) Enhanced activity by poly(ethylene glycol) modification of *Coriolopsis gallica* laccase. J Ind Microbiol Biotechnol 29:214–20

Varadachari V, Ghosh K (1984) On humus formation. Plant Soil 77:305–313

Vasdev K, Kuhad RC, Saxena RK (1995) Decolorization of triphenylmethane dyes by *Cyathus bulleri*. Curr Microbiol 30(5):269–272

Vasdev K, Kuhad RC (1994) Induction of Laccase production in *Cyathus bulleri* under shaking and static conditions. Folia Microbiol 39(4):326–330

Viswanath B, Chandra MS, Kumar KP, Rajasekhar-reddy B (2008) Production and purification of laccase from *Stereum ostrea* and its ability to decolorize textile dyes. Dyn Biochem Process Biotechnol Mol Biol 2:19–25

Waksman SA, Cordon TC, Hulpoi N (1939) Influence of temperature upon the microbiological population and decomposition processes in composts of stable manure. Soil Sci 47:83–114

Watanabe K (2001) Microorganisms relevant to bioremediation. Curr Opin Biotechnol 12:237–241

Wood J, Wood P (1992) Evidence that cellobiose: quinine oxidoreductase from *Phanerochaete chrysosporium* is

a breakdown product of cellobiose oxidase. Biochem Biophys Acta 1119:90–96

Wyatt AM, Broda P (1995) Informed strain improvement for lignin degradation by *Phanerochaete chrysosporium*. Microbiology 141:2811–2822

Xu F (2005) Applications of oxidoreductases: recent progress. Ind Biotechnol 1:38–50

Yamanaka R, Soares CF, Matheus DR, Machado KMG (2008) Lignolytic enzymes produced by *Trametes villosa* CCB 176 under different culture conditions. Braz J Microbiol 39:78–84

Yang XQ, Zhao XX, Liu CY, Zheng Y, Qian SJ (2009) Decolorization of azo, triphenylmethane and anthraquinone dyes by a newly isolated *Trametes* sp. SQ01 and its laccase. Process Biochem 44:1185–1189

Yaropolov AI, Skorobogat'ko OV, Vartanov SS, Varfolomeyev SD (1994) Laccase: properties, catalytic mechanism, and applicability. Appl Biochem Biotechnol 49(3):257–280

Zavarzina AG, Leontievsky AA, Golovleva LA, Trofimov SY (2004) Biotransformation of soil humic acids by blue laccase of *Panus tigrinus*: an *in vitro* study. Soil Biol Biochem 36:359–69

Zhao YC, Yi XY, Zhang M, Liu L, Ma WJ (2010) Fundamentals of degradation of dichlorodiphenyltrichloroethane in soil by laccase from white rot fungi. Int J Environ Sci Technol 7:359–366

Zille A, Tzanov T, Guebitz GM, Cavaco-Paulo A (2003) Immobilized laccase for decolourization of Reactive Black dyeing effluent. Biotechnol Lett 25:1473–1477

Microbial Phytases in Skirmishing and Management of Environmental Phosphorus Pollution

13

Sapna, Bijender Singh, Deepti Singh, and K.K. Sharma

Abstract

Phytases are acid phosphatases which catalyze the hydrolysis of phytic acid (*myo*-inositol hexakis(dihydrogen phosphate)) to inorganic phosphate and *myo*-inositol through a series of *myo*-inositol phosphate intermediates and making insoluble and unavailable phytic acid available for animal assimilation. Phytic acid is a major stored form of organic phosphorus in plants such as cereal grains, legumes and oilseeds. The accumulation of phosphorus at intensive livestock production area has raised serious concerns of environmental pollution. Phytases are of significant value in effectively combating environmental phosphorus pollution. The microbial sources of phytases include bacteria, moulds and yeasts. The supplementation of animal feeds with microbial phytases increases the bioavailability of phosphorus and minerals, besides reducing the aquatic phosphorus pollution in the areas of intensive livestock production.

Microbial phytases are produced by solid state and submerged fermentations. The molecular masses of microbial phytases are in the range of 35–500 kDa depending upon the source, and they are active within a pH and temperature ranges of 4.5–7.5 and 45–70°C. Phytases not only degrade phytic acid but also ameliorate the nutritional status of the foods by making minerals such as iron, magnesium, zinc, phosphorus and proteins available for monogastric animals. This chapter describes the sources, production, characterization and applications of microbial phytases in phosphorus pollution management.

Keywords

Phytase • Phytic acid • Anti-nutritional factor • Phosphorus • Environmental pollution • Peroxidase

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Introduction

Phosphorus is a major constituent in biosynthesis of nucleic acids and cell membranes with an important role in regulation of a number of enzymes. Phosphorus deficiency in soil is a major problem for agricultural production as most soils contain significant amounts of total soil phosphorus that occurs either as inorganic or organic fractions. Phytic acid as phytate (salts of phytic acid) is the major storage form of organic phosphorus in soil, and it is not readily available to plants as a source of phosphorus because of its complex with cations or adsorption to various soil components. Phosphorus is an important and essential nutrient to both plant and animal life but in excess can cause pollution. Phytic acid is the principal storage form of phosphorus which comprises 1–5% by weight in cereals, legumes, oil, seeds and nuts (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Greiner and Konietzny 2006; Rao et al. 2009; Singh et al. 2011; Singh and Satyanarayana 2011a, b). This phytate phosphorus is largely unavailable to monogastric animals (chicken, swine, fishes, human) due to either the absence or insufficient secretion of phytase in their gastrointestinal tract (Maenz and classen 1998; Boling et al. 2000; Singh and Satyanarayana 2011a, b). Therefore, phytic acid cannot be absorbed in the digestive tract and released in to the environment and cause pollution.

Excess amount of phosphorus causes rapid growth of phytoplanktons, algae, creating dense population of blooms (Richard et al. 1998). Due to these blooms plants cannot photosynthesize and produce food which they need to survive. Phytic acid can be removed by some physical (autoclaving, cooking) and chemical (ion exchange, acid hydrolysis) methods, but these methods decrease the nutritional value of the food (Vohra and Satyanarayana 2003; Singh et al. 2011). Therefore, the reduction of phytic acid content in food and feed by enzymatic action using phytase is desirable as it improves the nutritional value of the food (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Greiner and Konietzny 2006; Rao et al. 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). This enzyme also has potential applications in other fields and is of immense commercial value primarily in feed and food industries. The annual sale of commercial supplemental phytase is estimated as 150 million euro, which is one third of the entire feed enzyme market and is still increasing (Greiner and Konietzny 2006). Phytate phosphorus is an alternative economical source that can be effectively converted to available phosphate by phytases. Feeds for pigs and poultry are commonly supplemented with inorganic phosphate in order to fulfil their phosphorus requirement for optimal growth of animals (Lei and Stahl 2001; Singh et al. 2011).

Phytic acid is degraded by phytase that was first reported by Suzuki et al. in 1907. The first commercial preparation of phytase came to market in Europe in 1994 via Gist Brocades. Phytase hydrolyses phytic acid to *myo*-inositol and inorganic phosphate through a series of *myo*-inositol phosphate intermediates (Mitchell et al. 1997). Phytases are generally found in plants and microorganisms, whereas phytase activity of animals is negligible as compared to microbes (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Greiner and Konietzny 2006; Rao et al. 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). Most of the scientific work has been done on microbial phytases, especially on those originating from filamentous fungi such as *Aspergillus niger* and *Mucor piriformis* (Vats and Banerjee 2005; Vats et al. 2009); bacteria such as *Clostridium* spp., *Bacillus subtilis*, *Bacillus* sp. KHU-10, *B. laevolacticus* and *Klebsiella aerogenes* (Kerovuo et al. 1998; Gulati et al. 2007); and yeasts such as *Pichia anomala*, *Arxula adeninivorans* and *Candida krusei* (Sano et al. 1999; Vohra and Satyanarayana 2001; Quan et al. 2001; Kaur et al. 2010). Phytase supplementation in animal feed will not only improve the nutritive value of feed but will also reduce environmental

Table 13.1 Phytate content in plant-derived food ingredients

S. no.	Plant food/ingredients	Phytate (%)
Cereals		
1.	Rice (polished, cooked)	0.12–0.37
2.	Maize bread	0.43–0.82
3.	Wheat bread	0.32–0.73
4.	Rye bread	0.19–0.43
5.	Sourdough rye bread	0.01–0.03
6.	French bread	0.02–0.04
7.	Flour bread (70% wheat, 30% rye)	0.04–0.11
8.	Cornflakes	0.04–0.15
9.	Oat flakes	0.84–1.21
10.	Sorghum	0.59–1.18
11.	Oat porridge	0.69–1.02
Legume-based food		
12.	Green peas (cooked)	0.18–1.15
13.	Black beans (cooked)	0.85–1.73
14.	Soybeans	0.92–1.67
15.	Lentils (cooked)	0.21–1.01
16.	Peanuts	0.92–1.97
17.	Chickpea (cooked)	0.29–1.17
18.	Cowpea (cooked)	0.39–1.32
19.	Kidney beans (cooked)	0.83–1.34
20.	White beans (cooked)	0.96–1.39
Miscellaneous		
21.	Amaranth grain	1.12–2.34
22.	Buckwheat	0.92–1.62
23.	Soy protein isolate	0.24–1.31
24.	Sesame seeds (toasted)	3.93–5.72
25.	Soy protein concentrate	1.12–2.34

phosphorus pollution (Singh and Satyanarayana 2011a, b; Singh et al. 2011).

Phytic Acid in Nutrition

Phytic acid is the major storage form of phosphorus in cereals, legumes and oilseeds which affects the functional and nutritional properties of food ingredients (Maga 1982; Tyagi and Verma 1998; Singh et al. 2011) Phytic acid is *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) which occurs primarily as salts of mono- and divalent cations in cereal grains and legumes (Table 13.1). It acts as an anti-nutrition factor by chelating metal ions, forming complexes with protein, making them unavailable and inhibiting the activity of some enzymes (Maga 1982) (Fig. 13.1). It accumulates in seeds and grains during ripening along with other storage substances such as starch and lipids. In cereals and legumes, phytic acid accumulates in the aleurone particles and globoid crystals, respectively (Reddy et al. 1982; Tyagi and Verma 1998). Phytate also acts as a strong chelator for divalent metal cations and exists as metal-phytate complex in plants (Asada et al. 1969; Reddy et al. 1982). In seeds and grains, phytic acid plays an important role in phosphorus storage, as an energy store, as a source of cations and as a source of *myo*-inositol and also helps in initiating dormancy (Singh et al. 2011). Graf et al. (1987) suggested that phytic acid in seeds acts as a natural antioxidant during dormancy.

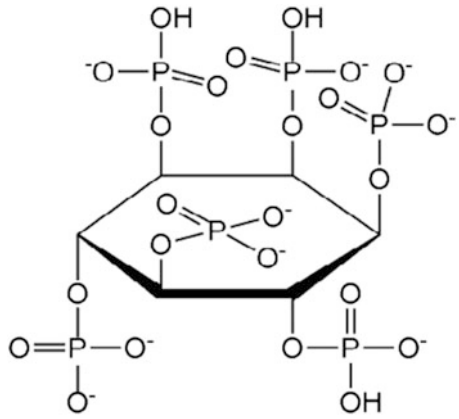

Fig. 13.1 Structure of phytic acid

Phytic acid has been shown to exert an anti-neoplastic effect in animal models of both colon and breast carcinomas. Undigested phytic acid present in the colon may protect against the development of colonic carcinoma (Iqbal et al. 1994). The inositol phosphate intermediates play an important role in the transport of materials into the cell, and inositol triphosphates play a role in signal transduction and regulation of cell functions in plant and animal cells (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Greiner and Konietzny 2006; Rao et al. 2009; Singh and Satyanarayana 2011a, b). Besides these functions, phytic acid also acts as an anti-nutritional factor in several ways due to the interactions with metal ions, proteins and enzymes.

Phytases

Phytases (*myo*-inositol hexaphosphate phosphohydrolase) hydrolyze phytic acid to *myo*-inositol and inorganic phosphates through a series of *myo*-inositol phosphate intermediates and eliminate its anti-nutritional properties (Mitchell et al. 1997; Vats and Banerjee 2004; Kaur et al. 2007; Fu et al. 2008; Rao et al. 2009; Singh et al. 2011). Phytase has been reported in microorganisms, plants and some animals (Wodzinski and Ullah 1996; Vohra and Satyanarayana 2003; Angelis et al. 2003; Vats and Banerjee 2004; Kaur et al. 2007; Fu et al. 2008; Rao et al. 2009; Raghavendra and Halami 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). A large number of fungi, bacteria and yeasts have been reported to produce phytase extra- and intracellularly as well as in the cell-bound form (Shieh and Ware 1968; Wodzinski and Ullah 1996; Pandey et al. 2001; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007; Fu et al. 2008; Rao et al. 2009; Raghavendra and Halami 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). There are two types of phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN):

(a) *Myo*-inositol-hexakisphosphate 3-phosphohydrolase, i.e. 3-phytase (EC 3.1.3.8), that first hydrolyses the ester bond at the 3 position of *myo*-inositol hexakisphosphate (Johnsen and Tate 1969) and is mainly reported in microorganisms.

(b) *Myo*-inositol-hexakisphosphate 6-phosphohydrolase, i.e. 6-phytase (EC 3.1.3.26), that first hydrolyses the ester bond at the 6 position of *myo*-inositol hexakisphosphate (Cosgrove 1969, 1970) and is mostly reported in plants. This had also been reported in some basidiomycetous fungi (Lassen et al. 2001). But an alkaline 5-phytase from lily pollen was found to start phytate hydrolysis at the D-5 position (Barrientos et al. 1994). On the basis of pH for activity, phytases can be broadly categorized into two major classes: acid phytases and alkaline phytases (Kaur et al. 2007; Singh et al. 2011). More focus has been on acidic phytases because of their applicability in animal feeds and broader substrate specificity than those of alkaline phytases. Recently, phytases have also been classified on the basis of their catalytic properties such as CP (cysteine phosphatase), BPP (β-propeller phytase), HAP (histidine acid phosphatase) and PAP (purple acid phosphatase) (Mullaney and Ullah 2003; Singh et al. 2011).

Sources and Production of Phytases

Phytases can be derived from various sources, including plants, animals and microorganisms. However, microbial sources are more promising for the production of phytases on a commercial scale (Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007; Fu et al. 2008; Rao et al. 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). The phytases of filamentous fungi are extracellular in nature (Mitchell et al. 1997; Singh and Satyanarayana 2006a, b, 2008a, b, c, 2011) unlike bacterial and yeast phytases, which are intracellular and cell-bound (Vohra and Satyanarayana 2002; Kaur and Satyanarayana 2010). A list of microbial sources and production conditions are given in Table 13.2.

Bacterial Phytases

Bacterial phytases have been reported in *Citrobacter freundii*, *Aerobacter aerogenes*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas* sp., *Selenomonas ruminantium*, *Enterobacter* sp., *Klebsiella oxytoca* MO-3 (Wodzinski and Ullah 1996; Choi et al. 2001; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Fu et al. 2008; Singh et al. 2011). Only *B. subtilis* (Powar and Jagannathan 1967) *and B. subtilis var. natto* (Shimizu 1992) were shown to produce extracellular phytase. The phytase production by *B. subtilis* was induced by phytate in the cultivation medium. It required Ca^{2+} ions for its activity (Powar and Jagannathan 1982). Also cytoplasmic phytase from *Klebsiella terrigena* is inducible in the presence of phytate under carbon limitation (Greiner et al. 1997). The activity of intracellular *E. coli* phytase was remarkably increased in the stationary phase and under anaerobic conditions (Greiner et al. 1993).

Mould Phytases

Microbial phytase activity was most frequently detected in filamentous fungi (Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007; Rao et al. 2009; Singh and Satyanarayana 2011a, b; Singh et al. 2011). Both solid-state fermentation (SSF) and submerged fermentation (SmF) have been used for phytase production by these microbes (Chadha et al. 2004; Singh and Satyanarayana 2006a, b, 2008a, b, c) (Table 13.2). Submerged fermentation is commercially used for phytase production, but recently scientists all over the world have focused their attention on SSF because of its advantages over SmF (Pandey et al. 1999, 2001). Among all the thermophilic fungal isolates, *R. pusillus* produced phytase maximally on wheat bran supplemented with basal medium, containing asparagine and corn steep liquor as nitrogen sources (Chadha et al. 2004). Shieh and Ware (1968) tested over 2,000 microorganisms isolated from soil but extracellular phytase activity was observed in only 30 isolates. All phytase producers were filamentous fungi belonging to the genera of *Aspergillus*, *Penicillium* and *Mucor*. Howson and Davis (1983) confirmed *A. niger* strains as the best producers of extracellular phytase while bacterial cultures produced only intracellular enzyme. The phytase from *Thermomyces lanuginosus* was also cloned and over-expressed (Berka et al. 1998). *Chaetomium thermophilum* ATCC 58420, *Rhizomucor miehei* ATCC22064, *Thermomucor indicae-seudaticae* ATCC28404, *Myceliophthora thermophila* ATCC48102 (Mitchell et al. 1997), *A. fumigatus* (Pasamontes et al. 1997b), *Thermoascus aurantiacus* (Nampoothiri et al. 2004), *T. lanuginosus* (Berka et al. 1998; Gulati et al. 2007) and *Sporotrichum thermophile* (Singh and Satyanarayana 2006a, b, 2008a, b, c,) are also known to produce extracellular phytases. Thermophilic mould *S. thermophile* produces phytase optimally at 45°C and at a pH of 6.0. The mould also secreted phytase in solid-state fermentation in 120 h at 45°C, at the initial substrate to moisture ratio of 1:2.5 and a_w of 0.95 (Singh and Satyanarayana 2006a), and 2.6-fold improvement was further achieved due to statistical optimization (Singh and Satyanarayana 2008b). The moulds also secreted phytase optimally in synthetic medium after 5 days at 45°C and 250 rpm. Starch, glucose, sodium phytate and peptone were identified as

Table 13.2 Microbial sources and production of phytate-degrading enzyme

Microorganisms	T_{opt} (°C)	pH_{opt}	Location[a]	Fermentation[b]	References
Bacteria					
Aerobacter aerogenes	27	6.8	CB	SmF	Greaves et al. (1967)
Bacillus subtilis	37	7.0	EX	SmF	Kerovuo et al. (1998)
Bacillus sp. *DS11*	37	6.5	EX	SmF	Kim et al. (1998)
B. amyloliquefaciens	37	6.8	EX	SmF	Idriss et al. (2002)
Escherichia coli	37	7.0	CB	SmF	Sunita et al. (2000)
Lactobacillus sanfranciscensis	37	5.5	IN	SmF	Angelis et al. (2003)
L. amylovorus	37	6.5	EX	SmF	Raghavendra and Halami (2009)
L. rhamnosus	37	6.5	IN	SmF	Raghavendra and Halami (2009)
Klebsiella aerogenes	30	7.0	CB	SmF	Tambe et al. (1994)
Pediococcus pentosaceus	37	6.5	IN	SmF	Raghavendra and Halami (2009)
Fungi					
Aspergillus niger van Tieghem	30	5.5	EX	SmF	Vats and Banerjee (2005)
A. oryzae	37	6.4	EX	SmF	Shimizu (1993)
A. carneus	30	6.0	EX	SmF	Ghareib (1990)
A. fumigatus SRRC 322	37	5.0	EX	SmF	Mullaney et al. (2000)
Thermomyces lanuginosus	37	6.0	EX	SSF	Berka et al. (1998)
Thermoascus aurantiacus	45	5.5	EX	SmF	Nampoothiri et al. (2004)
Rhizomucor pusillus	50	8.0	EX	SSF	Chadha et al. (2004)
Myceliophthora thermophila	45	5.5	EX	SmF	Mitchell et al. (1997)
Rhizopus oryzae	30	5.5	EX	SSF	Ramachandran et al. (2005)
R. oligosporus	27	5.5	EX	SmF	Casey and Walsh (2004)
Sporotrichum thermophile	45	5.0	EX	SmF and SSF	Singh and Satyanarayana (2006a, b, 2008a, b, c)
Yeast					
Arxula adeninivorans	28	5.5	EX	SmF	Sano et al. (1999)
Candida krusei	40	4.6	CB	SmF	Quan et al. (2001)
Hanseniaspora guilliermondii	30	6.5	–	SmF	Hellstrom et al. (2010)
Pichia anomala	25	6.0	CB	SmF	Vohra and Satyanarayana (2001)
P. spartinae	75	4.5	–	SmF	Nakamura et al. (2000)
P. rhodanensis	70	4.5	–	SmF	Nakamura et al. (2000)
Schwanniomyces occidentalis	60	4.5	EX	SmF	Nakamura et al. (2000)
S. castellii	77	4.4	EX	SmF	Segueilha et al. (1992)

[a]*IN* Intracellular, *EX* Extracellular, *CB* Cell-bound
[b]*SSF* Solid-state fermentation, *SmF* Submerged fermentation

most significant factors by Plackett–Burman design and these were further optimized by response surface methodology (Singh and Satyanarayana 2008a). *Thermoascus aurantiacus* TUB F43 synthesized phytase in a medium containing glucose and starch as carbon sources and peptone as a nitrogen source at 45°C, 150 rpm and pH 5.5 after 72 h of fermentation (Nampoothiri et al. 2004). *Aspergillus fumigatus* secreted a heat-stable phytase that was able to withstand temperatures up to 100°C over a period of 20 min (Pasamontes et al. 1997b). Pasamontes et al. (1997a) reported the cloning of a phytase gene from *Talaromyces thermophilus* that showed 61% sequence homology with that of *A. niger*. Hassouni et al. (2006) studied phytase production by *M. thermophila* in solid-state fermentation using sugarcane bagasse, and maximum

phytase production was achieved at 45°C and pH 6.0, after 36 h of incubation at a moisture level of 70%. Phytase production by the thermophilic mould *T. lanuginosus* TL-7 was optimized using wheat bran as a substrate in SSF using a Box–Behnken factorial design of response surface methodology, which resulted in maximum phytase production (Gulati et al. 2007).

Phytase production by *A. niger* NCIM 563 was affected by inorganic phosphate content of agriculture (Bhavsar et al. 2008). The agriculture residues containing less than 4 mg/g inorganic phosphate along with 1% rice bran supported high phytase production on the 11th day of fermentation. Addition of glucose up to 5% in fermentation medium further enhanced phytase production. Pretreatment of agriculture residues with water has significantly enhanced the phytase activity in various agro-residues. A hyper-producing strain of *A. niger* van Tieghem isolated from wood logs produced 184 nkat/ml phytase activity in minimal medium at 30°C and pH 6.5 (Vats and Banerjee 2002). Among the different carbon sources, a combination of glucose and starch supported high phytase production. Biopeptone and ammonium nitrate supported maximum enzyme yield while the specific activity of phytase was comparatively higher when ammonium nitrate was used as sole source of nitrogen. Regulation of the *A. niger* var. *ficuum* NRRL 3135 phytase production was first described by Shieh et al. (1969), and the culture medium was subsequently optimized by Han and Gallagher (1987). Kujawski and Zyła (1992) observed a correlation of the ability of *A. niger* to produce citric acid by the solid surface method with its ability to synthesize intracellular phytase and acid phosphatase. *A. niger* phytase was produced extracellular during active cell growth (Volfova et al. 1994). Chelius and Wodzinski (1994) increased extracellular *A. niger* NRRL 3135 phytase activity by a method involving irradiation of cell suspension.

Yeast Phytases

Phytase production by yeasts has also been studied and reviewed (Table 13.2) earlier (Vohra and Satyanarayana 2001, 2002, 2003, 2004; Vats and Banerjee 2004; Kaur et al. 2007; Vohra et al. 2011; Singh et al. 2011). Both conventional and nonconventional yeasts are known to secrete phytases (Nayini and Markakis 1984; Segueilha et al. 1992; Lambrechts et al. 1993; Vohra and Satyanarayana 2001). *Saccharomyces cerevisiae* was found to produce phytase (Nayini and Markakis 1984), and further *Candida tropicalis, Torulopsis candida, Debaryomyces castellii, Kluyveromyces fragilis* and *Schwanniomyces castellii* were able to hydrolyze phytate (Lambrechts et al. 1992). Yeast *S. castellii,* which secreted the highest phytase, was chosen for further study (Segueilha et al. 1992; Lamberchts et al. 1993). *Pichia anomala*, a yeast, isolated from dried flowers of *Woodfordia fruticosa*, produces a cell-bound phytase. The enzyme productivity was very high in fed-batch fermentation in air-lift fermenter as compared to that in stirred-tank fermenter. When the yeast cells were permeabilized with Triton-X-100, amelioration in the cell-bound phytase activity was observed (Vohra et al. 2011). Phytase from baker's yeast was first extracted by Nayini and Markakis (1984) at 45°C and pH 4.6. Interestingly, Vohra and Satyanarayana (2001) and Quan et al. (2001, 2002) reported the production of a cell-bound phytase from *Pichia anomala* and *Candida krusei* WZ-001, respectively. Recently, there was a report on phytase-producing marine yeasts (Li et al. 2008).

Arxula adeninivorans, a nonconventional yeast like *Pichia anomala*, was shown to assimilate phytate as a sole source of carbon and phosphate (Sano et al. 1999). This ability was associated with a high phytate activity secreted into the medium. A high level of phytase production was attained when the inoculum was raised on phytate minimal agar plate. Galactose was a preferred carbon source instead of glucose which increased phytase titers several folds. The phytase production was high when yeast was grown at 44°C (Sano et al. 1999). Phytase production by *S. cerevisiae* was achieved at 40°C and pH 3.6 (In et al. 2009). Hellstrom et al. (2010) isolated yeast like *Issatchenkia orientalis* and *Hanseniaspora guilliermondii*, which produce phytase at 30°C and pH 6.5.

Purification and Characterization of Phytases

Separation of a protein from the biological environment requires a series of purification steps, each step removing some of the impurities and bringing the product closer to the final specification. The choice of procedure for enzyme purification depends on their location. Initial processes for phytase purification include crude fractionation, clarification and concentration of crude enzyme using centrifugation, ultrafiltration and salt precipitation (Singh and Satyanarayana 2011a). The concentrate is then further purified using high-resolution techniques based on chromatographic and electrophoretic separations. Purification of enzymes are needed for studying their structure–function relationships and biochemical properties. Phytase of a thermophilic mould *S. thermophile* was purified to homogeneity using acetone precipitation followed by ion exchange and gel filtration chromatography (Singh and Satyanarayana 2009). The molecular mass of the native enzyme was ~456 kDa and it was a homopentameric protein. Phytases from *M. thermophila, T. thermophilus* and *E. nidulans* were purified by using ultrafiltration followed by gel filtration and ion exchange chromatography (Wyss et al. 1999b). Phytase of *R. pusillus* was partially purified using ion exchange and gel filtration chromatography (Chadha et al. 2004). Phytase produced by *T. lanuginosus* was also purified by a two-step process using ion exchange and gel filtration chromatography (Gulati et al. 2007). *Aspergillus fumigatus* WY-2 phytase expressed in *P. pastoris* was purified by ammonium sulphate precipitation followed by ion exchange chromatography using DEAE-Sepharose column (Wang et al. 2007). A phytase with a high affinity for phytic acid was found in *A. niger* SK-57 and was purified by using ion exchange chromatography, gel filtration and chromatofocusing (Nagashima et al. 1999). Phytase of *A. oryzae* NRRL was purified by acetone fractionation, gel filtration, followed by DEAE-cellulose chromatography (Wang et al. 1980). Phytase from *P. anomala* was purified by acetone precipitation and anion exchange chromatography (Vohra and Satyanarayana 2002). Phytase isolated from bacteria such as *B. subtilis* by using phenyl-sepharose Superose 12 and column chromatography (Powar and Jagannathan 1982). A novel phytase was purified from *A. ficuum* NTG-23 by ion-exchange chromatography on DEAE-cellulose, CM-cellulose and FPLC-gel filtration on Superdex 75 which exhibited a molecular mass of 65.5 kDa (Zhang et al. 2010). It was optimally active at pH of 1.3 and 67°C with a K_m and V_{max} values of 0.295 mM and 55.9 nmol (phosphate)/min, respectively. Phytase activity was not significantly affected by metal ions. The enzyme exhibited a broad substrate specificity and showed strong resistance towards pepsin and trypsin. The purification strategies for different microbial phytases have been given in Table 13.3. Phytase from *B. subtilis* (natto) was purified by gel filtration and DEAE chromatography (Shimizu 1992). Phytase of *Bacillus* sp. DS11 was purified by acetone precipitation and phenyl-sepharose chromatography (Kim et al. 1998).

Phytase activity is usually measured by the amount of inorganic phosphate released per minute from a selected substrate under certain pH and temperature. Just like other enzymes, phytase activity is affected by the inherent properties of the enzyme and the reaction conditions. Properties of enzymes are important in determining their potential applications in different industries. The following properties of phytase are of practical significance:

pH

Most microbial phytases, especially from fungal origin, show the optimum pH between 4.5 and 5.5; some bacterial ones have optimum pH at 6.5–7.5 (Table 13.2). For phytase of *A. aerogenes* (Greaves et al. 1967), *Pseudomonas* sp. (Irving and Cosgrove 1971), *E. coli* (Greiner et al. 1993), *S. ruminantium* (Yanke et al. 1999) and *L. amylovorus* (Sreeramulu et al. 1996), the optimum pH ranged from 4 to 5.5. The pH optimum of *Enterobacter* sp. 4 (Yoon et al. 1996) and *Bacillus*

Table 13.3 Properties of microbial phytate-degrading enzymes

Microorganisms	Purification process	T_{opt} (°C)	pH_{opt}	MW (kDa)	Substrate[a]	References
Bacteria						
Aerobacter aerogenes	–	25	4.0–5.0	–	–	Greaves et al. (1967)
Bacillus subtilis	Hydrophobic and gel filtration chromatography	60	7.5	37	PS	Powar and Jagannathan (1982)
B. subtilis (natto)	Gel filtration and DEAE chromatography	60	6.0–6.5	38	PS	Shimizu (1992)
Bacillus sp. *DS11*	Acetone precipitation, phenyl-sepharose	70	7.0	–	PS	Kim et al. (1998)
B. licheniformis	–	65	6.0–7.0	47	PS	Tye et al. (2002)
Escherichia coli	$(NH_4)_2SO_4$ precipitation, ion exchange and hydrophobic interaction chromatography	55	4.5	42	PS	Greiner et al. (1993)
Klebsiella oxytoca	–	55	5.0–6.0	40		Jareonkitmongkol et al. (1997)
K. aerogenes	Ion exchange and gel filtration chromatography	65	4.5	700	PS	Tambe et al. (1994)
Pseudomonas syringae	Ion exchange chromatography	40	5.5	47	PS	Cho et al. (2003)
S. ruminantium	Ultrafiltration	50–55	4.5–5.0	46	–	Yanke et al. (1999)
L. sanfrancisensis	–	45	4.0	50	–	Angelis et al. (2003)
Fungi						
Aspergillus fumigatus	Ultrafiltration	58	5.0	75	BSS	Mullaney et al. (2000)
A. niger SK-57	Ion exchange and gel filtration chromatography and chromatofocusing	50	5.5,2.5	60	BSS	Nagashima et al. (1999)
A. niger	–	55	2.5	353	BSS	Vats and Banerjee (2005)
A. carneus	Acetone precipitation, gel filtration and Ion exchange chromatography	40	5.6	–		Ghareib (1990)
A. oryzae	–	50	5.5	120–140	BSS	Shimizu (1993)
Rhizopus oligosporus	Ion exchange and gel filtration chromatography and chromatofocusing	65	5.0	124	BSS	Casey and Walsh (2004)
Peniophora lycii	–	50–55	4.0–4.5	72	–	Lassen et al. (2001)

(continued)

Table 13.3 (continued)

Microorganisms	Purification process	T_{opt} (°C)	pH_{opt}	MW (kDa)	Substrate[a]	References
Trametes pubescens	–	50	5.0–6.0	62	–	Lassen et al. (2001)
T. lanuginosus	–	65	7.0	60	BSS	Berka et al. (1998)
Rhizomucor pusillus	Lyophilization, ion exchange and gel filtration chromatography	70	5.4	–	BSS	Chadha et al. (2004)
Sporotrichum thermophile	Acetone precipitation, ion exchange and gel filtration chromatography	60	5.5	456	BSS	Singh and Satyanarayana (2009)
Yeast						
Arxula adeninivorans	Gel filtration and ion exchange chromatography	75	4.5	–	–	Sano et al. (1999)
C. krusei WZ001	Ion-exchange, hydrophobic interaction and gel filtration.	40	4.6	330	–	Quan et al. (2002)
Pichia anomala	Acetone precipitation, anion exchange chromatography	60	4.0	64	BSS	Vohra and Satyanarayana (2002)
P. rhodanensis	–	70–75	4.0–4.5	–	–	Nakamura et al. (2000)
P. spartinae	–	75–80	4.5–5.0	–	–	Nakamura et al. (2000)
Schwanniomyces castellii	Ion exchange and gel filtration chromatography	77	4.4	490	BSS	Segueilha et al. (1992)

[a]*PS* phytate specific, *BSS* broad substrate specific

sp. DS11 (Kim et al. 1998) phytase was in the neutral range. *Aspergillus niger* NRRL 3135 produced two different phytases, first phyA with pH optima at 5.5 and 2.5 and the other phyB at pH 2.0 (Howson and Davis 1983). Nakamura et al. (2000) studied a number of yeast isolates for extracellular phytase activity. All the yeast phytases were optimally active at pH of 4–5 with temperature optima of 50–60°C.

Temperature

Phytases with optimal activity at elevated temperature are desirable in animal feed industry because feed pelleting involves a step at 80–85°C for few seconds. Microbial phytases show diversity in their temperature optima (Table 13.2). Phytases from bacteria such as *B. subtilis* (Powar and Jagannathan 1982), *E. coli* (Greiner et al. 1993), *K. aerogenes* (Tambe et al. 1994), *Enterobacter* sp. 4 (Yoon et al. 1996), *K. oxytoca* MO-3 (Jareonkitmongkol et al. 1997), *Bacillus* sp. DS11 (Kim et al. 1998) and *S. ruminantium* (Yanke et al. 1998) were optimally active in the range of 50–70°C, while phytase of *A. aerogenes* had an optima at 25°C (Greaves et al. 1967). Phytase of *S. castellii* showed optimum activity at 77°C (Segueilha et al. 1992) and that of *A. adeninivorans* at 75°C (Sano et al. 1999). Phytases from *P. rhodanensis* and *P. spartinae* were optimally active in the range of 70–80°C (Nakamura et al. 2000). Among the thermophilic moulds, *T. lanuginosus* exhibited optimum phytase activity at 65°C (Berka et al. 1998) and *S. thermophile* at 60°C (Singh and Satyanarayana 2009). Phytase of *A. fumigatus* and *A. niger* NRRL 3135 showed optimum activity at 37°C (Pasamontes et al. 1997b) and at 55°C (Howson and Davis 1983), respectively.

Proteolysis Resistance

Under normal conditions phytase is degraded by proteases present in the digestive tract of the test animals. Therefore, an effective phytase needs to be resistant to the action of these proteases. Microbial phytases showed different sensitivities to pepsin and trypsin (Kerovuo et al. 1998; Rodriguez et al. 1999). Phytase from a thermophilic mould *A. fumigatus* WY-2 was insensitive to pepsin and it retained about 90.1% residual activity (Wang et al. 2007). HAP-phytase from *S. thermophile* was also resistant to the action of proteases (Singh and Satyanarayana 2006b).

Effect of Metal Ions

Phytases from microbes differed in their requirement of metal ions for their activity. The bacterial phytases are mostly metal dependent as compared to yeasts and filamentous fungal phytases (Kaur et al. 2007; Rao et al. 2009; Gulati et al. 2007; Singh et al. 2011). Phytase of *S. castellii* was slightly inhibited in the presence of 5 mM Ca^{2+} and Mg^{2+}, Mn^{2+} and Fe^{2+} (Segueilha et al. 1992). Phytase of *Bacillus* sp. DS11 was strongly inhibited by EDTA, Cd^{2+} and Mn^{2+} and moderately inhibited by Hg^{2+}, Mg^{2+}, Ba^{2+} and Cu^{2+} at 5 mM (Kim et al. 1998).

Molecular Weight

Microbial phytases are high-molecular-weight proteins ranging from 40 to 500 kDa (Table 13.3). Zymogram study of phytase of *S. ruminantium* suggested that its phytase had molecular mass of 46 kDa (Yanke et al. 1999). The molecular mass of *Bacillus* sp. DS11 phytase was estimated by gel filtration and electrophoresis (Kim et al. 1998). The electrophoresis under denaturation conditions revealed a single protein band of 44 kDa whose size corresponded well with the molecular mass of 40 kDa obtained by Superose 12 column chromatography. An extracellular phytase from *B. subtilis* (natto) N-77 was purified 322-fold by gel filtration and DEAE chromatography (Shimizu 1992). The molecular mass of the active monomeric form was determined as 36 kDa by SDS-PAGE. Phytase from *S. castellii* was purified by anion exchange and gel filtration chromatography (Segueilha et al. 1992). The phytase of *S. thermophile* was a 456 kDa homopentameric and

glycosylated enzyme (Singh and Satyanarayana 2009). The molecular masses of phytases of *E. nidulans* and *T. thermophilus* were 51.785 kDa and 51.45 kDa, respectively (Pasamontes et al. 1997a). The molecular mass of the phytase of *A. fumigatus* was 48.27 kDa while that of *T. lanuginosus* was 60 kDa (Berka et al. 1998).

Current and Potential Applications of Microbial Phytases

Up to now, phytases have been mainly used as a feed supplement in diets largely for swine and poultry, and to some extent for fishes. Numerous laboratory experiments and field trials have shown that addition of phytase can replace inorganic phosphorus supplementation and reduce total phosphorus excretion by 30–50% (Yi et al. 1996; Kemme et al. 1997). Thus, the benefits of phytase are manyfold:

1. Saving the expensive and nonrenewable inorganic phosphorus resource by reducing the need for its inclusion in animal diets
2. Protecting the environment from pollution of excessive manure phosphorus runoff
3. Improving the nutritional status of feeds by making metals and proteins available for the animals

Several dietary factors may reduce or enhance phytase-feeding efficacy. High levels of dietary calcium or calcium and phosphorus ratios reduce the effectiveness of phytase (Lei et al. 1994; Sandberg et al. 1993). Moderate to high levels of inorganic phosphorus may inhibit the full function of phytase. Although phytase was initially shown to hydrolyze phytate phosphorus in diets for chicks 30 years ago (Nelson et al. 1971), the commercial application had not been feasible for many years, due to the low enzyme yield and the high cost of the conventional phytase fermentation system. A large portion of world population ingests a high level of phytate from the plant staple foods, suffering from iron and zinc deficiencies (Tatala et al. 1998). Effectiveness of supplemental phytases in reducing the phytate content of legume-derived food products (Greiner and Konietzny 1999; Fredrikson et al. 2001) and improving nutritional availability of zinc and iron from plant-based diets to animals have been investigated and reviewed in detail (Stahl et al. 1999; Selle and Ravindran 2007; Pillai et al. 2009).

Terrestrial Pollution Management

Phosphorus is a necessary ingredient for animal and plant productivity. Too much or too little phosphorus can be a problem both for animal production and the environment. Scientists and researchers all over the world are finding ways for better utilization of phosphorus by monogastric animals, thus to increase its productive efficiency and at the same time to protect the environment. The poultry industry, which contributes more than $12 billion annually to the economy, generate enormous amount of manure annually, which must be disposed off in an eco-friendly manner. Generally this waste is used as a fertilizer in agriculture production. But the unutilized and excess phosphorus can enter into rivers and lakes by running water causing severe health, environmental and economic problems (Murugesan et al. 2005; Pillai et al. 2009).

Ruminant animals sustain the microflora that hydrolyzes phytic acid. However, monogastric animals such as humans, chickens and pigs produce little or no phytase in their digestive tract. Hence, the phytic acid phosphorus is unavailable and is excreted unutilized (Mullaney et al. 2000). Phytic acid present in the manure of these animals is enzymatically degraded by soil- and water-borne microorganisms. This phosphorus is transported into the water bodies causing eutrophication which is responsible for oxygen depletion in the water bodies. Pretreatment of animal feed with phytases would increase the availability of inorganic phosphorus, thereby improving the nutritional status of food and also help in combating phosphorus pollution (Singh et al. 2006, 2011). High-density livestock production has led to the leakage of pollutants into the environment, resulting in public concern about their effects. The global livestock population is estimated to be close to 4 billion animals that produced around

500 million tons of manure annually (Graham et al. 2003). This is expected to increase in the future due to increase in demand of meat by human population. The problem of manure disposal is also increasing day by day due to this increment. Manure volume, manure nitrogen and phosphorus contents, methane production and odour are the primary environmental factors (Jongbloed and Lenis 1998). Legislation in many regions has restricted the amount of manure that has to be applied per hectare in order to prevent environmental pollution (Graham et al. 2003). The number of strategies which the animal production industry should take into consideration to reduce environmental impact includes improving the efficiency of conversion of feed into edible products, reducing feed wastage and formulating diets that more closely satisfy the need of an animal. About 50–80% of the nitrogen and phosphorus are not utilized by animals but are excreted into the environment (Graham et al. 2003).

Mainly pigs and chickens are used in studies to improve nutrient efficiency to reduce excretion of environmental contaminants (Selle and Ravindran 2007). Addition of feed supplements and modifying feeding programmes to improve nutrient efficiency can result in significant decreases in the nitrogen, phosphorus, odour and dry matter weight of manure. The excretion of phosphorus can be reduced by 30% by replacing feed phosphate with phytase and by equally calculated digestible phosphorus content (Selle and Ravindran 2007). Application of phytase in all the pig and poultry feed in the Netherlands has resulted in reduced phosphate content in the manure. Microbial phytase also improves the apparent absorption of magnesium, zinc, copper and iron in pigs (Selle and Ravindran 2007). Phytase addition improved the concentration of magnesium, phosphorus, calcium, manganese and zinc in plasma, bone and the whole body (Vielma et al. 2004). Yan and Reigh (2002) demonstrated that the phytase supplementation increased the retention of calcium, phosphorus and manganese by catfish. The phytase supplementation in the diets significantly improved the digestibility of minerals, total phosphorus, phytate phosphorus and gross energy (Cheng and Hardy 2002). The experimental studies in animals and human have shown that phytic acid-rich diets can cause zinc deficiency. Phytic acid does not inhibit copper absorption, but has a modest inhibitory effect on manganese absorption. The inclusion of phytase to broiler diets increased the coefficient of phosphorus retention and reduced the presence of this element in poultry birds, thus, indicating a favourable environmental effect (Ahmad et al. 2000; Brenes et al. 2003; Vohra and Satyanarayana 2003; Juanpere et al. 2004; Murugesan et al. 2005; Vohra et al. 2006; Ahmadi et al. 2008; Pillai et al. 2009).

Aquatic Pollution Management

Water quality of the world is threatened by contamination with nutrients, primarily nitrogen and phosphorus which have been excreted by monogastric animals. Animal manure can be a valuable resource for farmers, providing nutrients, improving soil structure and increasing vegetative cover to decrease erosion potential. At the same time, application of manure nutrients in excess of crop requirements can result in environmental contamination. Environmental concerns with phosphorus are primarily associated with pollution of rivers and lake water. This pollution may be caused by runoff of phosphorus when application to land is in excess of crop requirements. The environmental impact assessment of the aquaculture industry is getting increasing attention all over the world. Farmers engaged in freshwater aquaculture and coastal marine operations are facing increasing pressure from various organizations to control farm discharge into the surrounding ecosystems. This discharge, particularly phosphorus loading, leads to eutrophication. The phosphorus in the feed ingredients occurs in a number of forms. It occurs in the inorganic form as well as phosphate complexes of protein, lipid and carbohydrate. These forms are available to the fish. Organic phosphorus (phytic acid) present in most grain and seed by-products is generally unavailable to fishes and monogastric animals as mentioned earlier. Fish excrete this organic phosphorus in soluble and

particulate forms. The water quality is directly affected by soluble and organic forms of phosphorus. However, the particulate forms accumulate in the sludge and the phosphorus is released slowly to the water. Dissolved form of phosphorus is the most important factor affecting water quality, because it is most available for phytoplankton growth. Microbial phytase supplementation in the diet of fish can overcome this problem. It makes the chelated phosphorus available to fish and hence there is less faecal excretion, thereby reducing environmental pollution. The feeding experiments have shown an improvement in the growth of fishes fed on phytase supplemented feeds (Sajjadi and Carter 2004; Singh et al. 2006, 2011). The environmental benefits of using this enzyme in fish feeds will be reduced requirement of mineral supplements, thereby reducing chances of excess inorganic phosphorus getting into the aquatic environment. The feeding trials with the animals have showed an improvement in their growth with phytase supplemented feeds (Baruah et al. 2005; Revy et al. 2006; Singh et al. 2006, 2011). Dietary phytase also improves the nutritive value of canola protein concentrate and decreases phosphorus output in a study performed with rainbow trout (Forster et al. 1999). Similar reports have been documented for different species like rainbow trout (Rodehutscord and Pfeiffer 1995), channel catfish (Li and Robinson 1997), African catfish (Van Weerd et al. 1999), common carp (Schafer et al. 1995) and *Pangasius pangasius* (Debnath et al. 2005). Robinson et al. (2002) reported that 250 units of phytase per kilogram of diet could effectively replace dicalcium phosphate supplement in the diet of channel catfish without affecting growth, feed efficiency or bone phosphorus deposition. The treatment of fish feed with phytase was found to improve protein digestibility and retention in fishes (Boling et al. 2001; Cheng and Hardy 2002; Usmani and Jafri 2002; Vielma et al. 2004; Sajjadi and Carter 2004; Debnath et al. 2005; Baruah et al. 2005; Ai et al. 2007; Altaff et al. 2008; Hassan et al. 2009). Use of phytase in feeds reduces or sometimes eliminates the necessity of mineral supplementation, which also decreases the cost of feeds.

Transgenic Approaches for Skirmishing Environmental Phosphorus Pollution

The high phosphorus content of manure from monogastric animals is a leading source of phosphorus pollution in the areas of intensive livestock production. Thus, using phytase as a feed additive is a more practical and environmentally sound approach, but its use is limited by the production cost, inactivation at high temperatures required for feed pelleting and loss of activity during storage. Golovan et al. (2001) approached this problem by an idea that if phytase is endogenously produced in the digestive tract of poultry, it may increase the bioavailability of plant phytate besides reducing the phosphorus output from animal production. Theoretically, an 'ideal' phytase should be catalytically efficient, proteolysis resistant, thermostable, cheap and broad substrate specific with longer shelf life (Lei and Stahl 2001). But, to the best of our knowledge none of the phytases studied so far have all these desired properties. However, single or multiple traits of phytases have been successfully improved by genetic manipulations. Some examples for transgenic modifications are:

1. Transgenic mice expressed *E. coli* phytase in salivary gland and secreted a biologically active 55-kDa, with low pH optimum, protease-resistant, glycosylated protein in saliva. The transgenes were regulated either by the inducible rat R15 proline-rich protein (PRP) promoter or by the constitutive mouse parotid secretory protein (PSP) promoter. Expression of salivary phytase leads to significant reduction of faecal phosphorus, which suggested it to be a promising approach to reduce requirements for dietary phosphorus supplementation and phosphorus pollution from animal manure.
2. Golovan et al. (2001) developed phytase transgenic pigs expressing salivary phytase to address the problem of manure-based environmental pollution in the pork industry. The salivary phytase has resulted in complete digestion of dietary phytate with concomitant omission of the inorganic phosphate supplementation and reduction of faecal phosphorus as compared

to wild pigs. Thus, this offered a unique biological approach for the management of phosphorus nutrition and environment pollution in the pork industry.

3. With the development of heterologous microbial expression systems, large amounts of the enzyme could be produced for animal feed use at relatively low costs. Submerged or solid-state fermentation of phytase over produces good yields of phytase at low cost (Pandey et al. 2001; Singh and Satyanarayana 2006a, 2008b). Recently, a great research effort has been made towards the use of methylotrophic yeast (*Pichia pastoris, Hansenula polymorpha*) (Han and Lei 1999; Wyss et al. 1999a, b; Rodriguez et al. 2000). Phytase has also been expressed in *Streptomyces lividans* (Stahl et al. 2003) and *L. plantarum* (Kerovuo and Tynkkynen 2000). The latter expression system offers a possibility of combining phytase with the beneficial probiotic lactic acid bacteria. A fungal phytase has been successfully expressed in seeds of soybean and alfalfa (Li et al. 1997; Ullah et al. 2002).

Miscellaneous Application of Phytases

(a) *Food and Feed Industry*
Phytase has degraded phytic acid during the manufacture of roller dried complementary foods based on flours from rice, wheat, maize, oat, sorghum, and a wheat–soy flour blend (Hurrell et al. 2003). Haros et al. (2001) investigated the possible use of phytases in the process of bread making. Different amounts of fungal phytase were added in whole wheat breads which proved phytase as an excellent bread-making improver. The fermentation period was shortened without affecting the bread dough pH. An increase in bread volume and an improvement in crumb texture were also observed.

(b) *Preparation of myo-Inositol Phosphates*
Inositol phosphates are known to play an important role in transmembrane signalling and mobilization of calcium from intracellular reserves resulted in the need for various *myo*-inositol phosphate preparations (Billington 1993). Phytases from microbial sources are known to produce different inositol phosphate intermediates. D-*myo*-inositol 1,2,6-triphosphate, D-*myo*-inositol 1,2,5-triphosphate, L-*myo*-inositol 1,3,4-triphosphate and *myo*-inositol 1,2,3-triphosphate were produced by hydrolysis of phytic acid by phytase of *S. cerevisiae* (Siren 1986a). Greiner and Konietzny (1996) prepared *myo*-inositol 1,2,3,4,5-pentakisphosphate, *myo*-inositol 2,3,4,5-tetrakisphosphate, *myo*-inositol 2,4,5-triphosphate and *myo*-inositol 2,5-biphosphate using immobilized phytase from *E. coli.* Inositol phosphate derivatives can be used as enzyme stabilizers (Siren 1986b), enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potential drugs and as chiral building blocks (Laumen and Ghisalba 1994). Phytase for converting phytic acid to lower *myo*-inositol phosphate derivatives or free *myo*-inositol and inorganic phosphate was suggested for the industrial production of *myo*-inositol or *myo*-inositol phosphates (Brocades 1991). Among the advantages of enzymatic hydrolysis are the stereo specificity and mild reaction conditions.

(c) *Plant Growth Promotion*
Phosphorus deficiency in soil is a major worldwide problem for agricultural production (Singh and Satyanarayana 2011b). Microorganisms play an important role in the acquisition and transfer of nutrients in soil. Soil phosphorus is present in inorganic and organic forms. The major organic form is phytates, i.e. salts of phytic acid. These salts are insoluble and are not available for assimilation by plants (Singh and Satyanarayana 2011b). However, microorganisms can solubilize and mineralize phosphorus from inorganic and organic source (Singh and Satyanarayana 2011b). There are a large number of reports on the role of phytase in improving the growth of plants and reducing the phosphorus pollution. Several *Bacillus* strains belonging to the *B. subtilis/amyloliquefaciens* isolated from rhizospheric soil were able to degrade phytate (Krebs et al. 1998). The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain

stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. Both phytase and mould promote the growth of wheat seedlings when phytic acid was supplied as sole source of phosphorus (Singh and Satyanarayana 2010). The growth and inorganic phosphate content of plants were better than control plants supplied with inorganic phosphate. The enzyme dose of 20 U per plant was sufficient to support plant growth. Plant growth, root/shoot length and the inorganic phosphate content of test plants were higher than control (Singh and Satyanarayana 2010). Yip et al. (2003) showed that the phytase transgenic tobacco plants showed phenotypic changes in flowering, seed development, and response to phosphate deficiency. The transgenic line showed an increase in number of flower and fruit, lesser seed IP6/IP5 ratio, and enhanced growth under phosphate-starvation conditions as compared to the wild type. Phytase and phosphatases producing fungi were used as seed inoculants to help attain higher phosphorus nutrition of plants in the soils containing high phytate phosphorus (Yadav and Tarafdar 2003). Transgenic *Arabidopsis* harbouring phytase gene of *Medicago truncatula* led to significant improvement in organic phosphorus utilization and plant growth (Xiao et al. 2005). Using phytate as the sole source of phosphorus, dry weight and total phosphorus content of the transgenic *Arabidopsis* lines were many fold higher than the control plants.

(d) *Peroxidase Activity of Phytases*

Microbial phytases share some active site homology with each other. If a vanadium ion is incorporated in the active site of a phytase, it behaves like a peroxidase (Singh et al. 2011). A semisynthetic peroxidase was designed by exploiting the structural similarity of the active sites of vanadium-dependent peroxidases and acid phosphatases. Under reaction conditions, the semisynthetic vanadium peroxidase is stable for over 3 days with only a slight decrease in turnover frequency (de Velde et al. 2000). Phytases from *A. ficuum*, *A. fumigatus* and *A. nidulans* catalyzed enantioselective oxygen transfer reactions when incorporated with vanadium. However, phytase from *A. ficuum* was unique in also catalyzing the enantioselective sulphoxidation, but at a lower rate, in the absence of vanadate ion. A cross-linked enzyme aggregate of 3-phytase was transformed into peroxidase by vanadate incorporation in the active site of the enzyme (Correia et al. 2008).

Conclusions and Future Perspectives

Phosphorus pollution in the area of intensive livestock production is the main area of concern for both food industrialist and bio-entrepreneurs. The livestock animals mainly feed on plant-derived ingredients which contain high amount of anti-nutritional factor, i.e. phytic acid. This anti-nutritional factor can be degraded by physical and chemical methods, but these methods results in loss of nutritional quality of food and feed. The non-ruminant animals do not sustain intestinal microflora which can degrade phytic acid; therefore, there is a necessity of supplementation of animal feed with microbial phytases. Microbial phytases not only degrade phytic acid but also improve the nutritional quality of food and feed by making minerals, phosphorus and proteins available to the animals.

Modern-day technology of molecular and genetic engineering has helped the scientist to develop transgenic microbes, animals and plants harbouring foreign genes of interest. Supplementation of microbial phytase to animal feed and generation of transgenic animals such as pigs and rats as well as plants maize and Arabidopsis-containing phytase gene have helped in the management of aquatic and terrestrial phosphorus pollution due to the reduction of phytic acid content and excretion to the environment. Therefore, supplementation of animal feed with microbial phytases will help in the skirmishing and management of environmental phosphorus pollution caused by the excess phosphorus and nitrogen present in the excreta of non-ruminant animals.

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References

Ahmad T, Rasool S, Sarwar, Haq A, Hasan Z (2000) Effect of microbial phytase produced from a fungus *Aspergillus niger* on bioavailability of phosphorus and calcium in broiler chickens. Anim Feed Sci Technol 83:103–114

Ahmadi A, Tabatabaei MM, Aliarabi H, Saki AA, Siyar SA (2008) Performance and egg quality of laying hens affected by different sources of phytase. Pak J Biol Sci 11:2286–2288

Ai Q, Mai K, Zhang W, Xu W, Tan B, Zhang C, Li H (2007) Effects of exogenous enzymes (phytase, non-starch polysaccharide enzyme) in diets on growth, feed utilization, nitrogen and phosphorus excretion of Japanese seabass, *Lateolabrax japonicus*. Comp Biochem Physiol 147:502–508

Altaff K, Hassan S, Satyanarayana T (2008) Use of phytase in plant based feed for aquaculture industry: cost effective and eco-friendly practice: present scenario and future perspectives. J Aquat Biol 23:185–193

Angelis MD, Gallo G, Corbo MR, McSweeney PLH, Faccia M, Giovine M, Gobbetti M (2003) Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. Int J Food Microbiol 87:259–270

Asada K, Tanaka K, Kasai Z (1969) Formation of phytic acid in cereal grains. Ann N Y Acad Sci 165:801–814

Barrientos L, Scott JJ, Murthy PP (1994) Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. Plant Physiol 106:1489–1495

Baruah KP, Sahu AK, Jain NP, Mukherjee KK, Debnath SC (2005) Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. Aquacult Res 36:803–812

Berka RM, Rey MW, Brown KM, Byun T, Klotz AV (1998) Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl Environ Microbiol 64:4423–4427

Bhavsar K, Shah P, Soni SK, Khire JM (2008) Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. Afr J Biotechnol 7(8):1101–1106

Billington WD (1993) Species diversity in the immunogenetic relationship between mother and fetus: is trophoblast insusceptibility to immunological destruction the only essential common feature for the maintenance of allogeneic pregnancy? Exp Clin Immunogenet 10(2):73–84

Boling SDD, Johnson MW, Wang ML, Parsons X, Koelkebeck CM, Zimmerman KW (2000) The effects of dietary available phosphorus levels and phytase performance of young and older laying hens. Poult Sci 79:224–230

Boling-Frankenbach SD, Peter CM, Douglas MW, Snow JL, Parsons CM, Baker DH (2001) Efficacy of phytase for increasing protein efficiency ratio values of feed ingredients. Poult Sci 80:1578–1584

Brenes A, Viveros A, Arija I, Centeno C, Pizarro M, Bravo C (2003) The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. Anim Feed Sci Technol 110:201–219

Brocades (1991) DNA sequence encoding phytase. Patent EP 420, 358

Casey A, Walsh G (2004) Identification and characterization of a phytase of potential commercial interest. J Biotechnol 110(3):313–322

Chadha BS, Gulati H, Minhas M, Saini HS, Singh N (2004) Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J Microbiol Biotechnol 20:105–109

Chelius MK, Wodzinski RJ (1994) Strain improvement of *Aspergillus niger* for phytase production. Appl Microbiol Biotechnol 41:79–83

Cheng ZJ, Hardy RW (2002) Effect of microbial phytase on apparent nutrient digestibility of barley, canola meal, wheat and wheat middlings, measured in vivo using rainbow trout (*Oncorhynchus mykiss*). Aquacult Nutr 8:271–277

Cho JS, Lee CW, Kang SH, Lee JC, Bok JD, Moon YS, Lee HG, Kim SC, Choi YJ (2003) Purification and characterization of a phytase from *Pseudomonas syringae* MOK1. Curr Microbiol 47(4):290–294

Choi YM, Suh HJ, Kim JM (2001) Purification and properties of extracellular phytase from *Bacillus* sp. KHU-10. J Protein Chem 20(4):287–292

Correia I, Aksu S, Adao P, Pessoa JC, Sheldon RA, Arends IWCE (2008) Vanadate substituted phytase: immobilization, structural characterization and performance for sulfoxidations. J Inorg Biochem 102:318–329

Cosgrove DJ (1969) Ion exchange chromatography of inositol polyphosphates. Ann N Y Acad Sci 165:677–686

Cosgrove DJ (1970) Inositol phosphates of microbial origin. Inositol phosphate intermediates in the dephosphorylation of the hexaphosphates of myoinositol, scyllo-inositol, and D-chiro-inositol by a bacterial (*Pseudomonas* sp.) phytase. Aust J Biol Sci 23:1207–1220

de Velde FV, Lars Konemann K, Rantwijk FV, Sheldon RA (2000) The rational design of semisynthetic peroxidases. Biotechnol Bioeng 67(1):87–96

Debnath D, Pal AK, Sahu NP, Jain KK, Yengkokpam S, Mukherjee SC (2005) Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. Aquacult Res 36:180–187

Forster I, Higgs DA, Dosanjh BS, Rowshandeli M, Parr J (1999) Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11°C fresh water. Aquacult Nutr 179:109–125

Fredrikson M, Biot P, Alminger LM, Carlsson NG, Sandberg AS (2001) Production process for high-quality pea-protein isolate with low content of oligosaccharides and phytate. J Agric Food Chem 49:1208–1212

Fu S, Sun J, Qian L, Li Z (2008) *Bacillus* phytases: present scenario and future perspectives. Appl Biochem Biotechnol 151(1):1–8

Ghareib M (1990) Biosynthesis, purification and some properties of extracellular phytase from *Aspergillus carneus*. Acta Microbiol Immunol Hung 37(2):159–164

Golovan SP, Hayes MA, Phillips JP, Forsberg CW (2001) Transgenic mice expressing bacterial phytase as a model for phosphorus pollution control. Nat Biotechnol 19:429–433

Graf E, Empson KI, Eaton JW (1987) Phytic acid: a natural antioxidant. J Biol Chem 262:11647

Graham H, Simmins PH, Sands J (2003) Reducing environmental pollution using animal feed enzymes. Commun Agric Appl Biol Sci 68:285–289

Greaves MP, Anderson G, Webley DM (1967) The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. Biochim Biophys Acta 132:412–418

Greiner R, Konietzny U (1996) Construction of a bioreactor to produce special breakdown products of phytate. J Biotechnol 48:153–159

Greiner R, Konietzny U (1999) Improving enzymatic reduction of *myo*-inositol phosphates with inhibitory effects on mineral absorption in black beans (*Phaseolus vulgaris* var. *preto*). J Food Process Preserv 23:249–261

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44:125–140

Greiner R, Konitzny U, Jany KD (1993) Purification and characterization of two phytases from *Escherichia coli*. Arch Biochem Biophys 303:107–113

Greiner R, Haller E, Konietzny U, Jany KD (1997) Purification and characterization of a phytase from *Klebsiella terrigena*. Arch Biochem Biophys 341:201–206

Gulati HK, Chadha BS, Saini HS (2007) Production, purification and characterization of thermostable phytase from thermophilic fungus *Thermomyces lanuginosus* TL-7. Acta Microbiol Immunol Hung 54:121–138

Han YW, Gallagher DJ (1987) Phytase production by *Aspergillus ficuum* on semi-solid substrate. J Ind Microbiol 2:195–200

Han YW, Lei XG (1999) Role of glycosylation in functional expression of an *Aspergillus niger* phytase (phyA) in *Pichia pastoris*. Arch Biochem Biophys 364:83–90

Haros M, Rosell CM, Benedito C (2001) Fungal phytase as a potential breadmaking additive. Eur Food Res Technol 213(4–5):317–322

Hassan S, Altaff K, Satyanarayana T (2009) Use of soybean meal supplemented with cell bound phytase for replacement of fish meal in the diet of juvenile milkfish, *Chanos chanos*. Pak J Nutr 8:341–344

Hassouni H, Ismaili-Alaoui M, Gaime-Perraud I, Augur C, Roussos S (2006) Effect of culture media and fermentation parameters on phytase production by the thermophilic fungus *Myceliophthora thermophila* in solid state fermentation. Micol Apl Int 18(2):29–36

Hellstrom AM, Vazques-juarez R, Svanberg U, Andlid TA (2010) Biodiversity and phytase capacity of yeasts isolated from Tanzanian togwa. Int J Food Microbiol 136:352–358

Howson SJ, Davis RP (1983) Production of phytate hydrolyzing enzymes by some fungi. Enzyme Microb Technol 5:377–382

Hurrell RF, Reddy MB, Juillerat M, Cook JD (2003) Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. Am J Clin Nutr 77:1213–1219

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borriss R (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology 148(Pt 7):2097–2109

In MJ, Seo SW, Kim DC, Oh NS (2009) Purification and biochemical properties of an extracellular acid phytase produced by the *Saccharomyces cerevisiae* CY strain. Process Biochem 44:122–126

Iqbal TH, Lewis KO, Cooper BT (1994) Phytase activity in the human and rat small intestine. Gut 35:1233–1236

Irving GCJ, Cosgrove DJ (1971) Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. Aust J Biol Sci 24:547–557

Jareonkitmongkol S, Ohya M, Watanbe R, Takagi H, Nakamori S (1997) Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3. J Ferment Bioeng 83:393–394

Jongbloed AW, Lenis NP (1998) Environmental concerns about animal manure. J Anim Sci 76:2641–2648

Jonson LF, Tate ME (1969) The conformational analysis of phytic acid based on NMR spectra. J Chem 47:63–73

Juanpere J, Pérez-Vendrell AM, Brufau J (2004) Effect of microbial phytase on broilers fed barley-based diets in the presence or not of endogenous phytase. Anim Feed Sci Technol 115:265–279

Kaur P, Satyanarayana T (2010) Improvement in cell-bound phytase activity of *Pichia anomala* by permeabilization and applicability of permeabilized cells in soymilk dephytinization. J Appl Microbiol 108(6):2041–2049

Kaur P, Kunze G, Satyanarayana T (2007) Yeast phytases: present scenario and future perspectives. Crit Rev Biotechnol 27:93–109

Kaur P, Singh B, Böer E, Straube N, Piontek M, Satyanarayana T, Kunze G (2010) Pphy-a cell-bound phytase from the yeast *Pichia anomala*: molecular cloning of the gene PPHY and characterization of the recombinant enzyme. J Biotechnol 149(1–2):8–15

Kemme PA, Jongbloed AW, Mroz Z, Beynen AC (1997) The efficacy of *Aspergillus niger* phytase in rendering phytate phosphorus available for absorption in pigs is influenced by pig physiological status. J Anim Sci 75:2129–2138

Kerovuo J, Tynkkynen S (2000) Expression of *Bacillus subtilis* phytase in *Lactobacillus plantarum*. Lett Appl Microbiol 30:325–329

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998) Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64:2079–2085

Kim YO, Kim HK, Bae KS, Yu JH, Oh TK (1998) Purification and properties of thermostable phytase from *Bacillus* sp. DS11. Enzyme Microb Technol 22:2–7

Krebs B, Hoding B, Kubart SM, Workie A, Junge H, Schmiedeknecht G, Grosch R, Bochow H, Hevesi M (1998) Use of *Bacillus subtilis* as biocontrol agent. 1. Activities and characterization of *Bacillus subtilis* strains. J Plant Dis Prot 105:181–197

Kujawski M, Zyła K (1992) Relationship between citric acid production and accumulation of phytate-degrading enzymes in *Aspergillus niger* mycelia. Acta Microbiol Pol 41(3–4):187–191

Lamberchts C, Boze H, Segueilha L, Moulin G, Galzy P (1993) Influence of culture conditions on the bit syntheses of *Schwanniomyces castellii* phytase. Biotechnol Lett 15:399–404

Lambrechts C, Boze H, Moulin G, Galzy P (1992) Utilization of phytate by some yeasts. Biotechnol Lett 14:61–66

Lassen SF, Breinholt J, Ostergaard PR, Brugger R, Bischoff A, Wyss M et al (2001) Expression, gene cloning and characterization of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp., and *Trametes pubescens*. Appl Environ Microbiol 67:4701–4707

Laumen K, Ghisalba O (1994) Preparative scale chemo enzymatic synthesis of optically pure D-myo-inositol-1-phosphate. Biosci Biotechnol Biochem 58:2046–2049

Lei XG, Stahl CH (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Microbiol Biotechnol 57:474–481

Lei XG, Ku PK, Miller ER, Yokoyama MT, Ullrey DE (1994) Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean meal diets of weanling pigs. J Anim Sci 72:139–143

Li MH, Robinson EH (1997) Microbial phytase can replace inorganic phosphorus supplements in channel catfish *Ictalurus punctatus*. J World Aquacult Soc 28:402–406

Li J, Hegeman CE, Hanlon RW, Lacy GH, Denbow DM, Grabau EA (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol 114:1103–1111

Li XY, Liu ZQ, Chi ZM (2008) Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: optimization by response surface methodology. Bioresour Technol 99:6386–6390

Maenz DD, Classen HL (1998) Phytase activity in small intestine brush-border membrane of the chicken. Poult Sci 77:557–564

Maga JA (1982) Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. Crit Rev Food Sci Nutr 16:1–48

Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, van Loon APGM (1997) The phytase subfamily of histidine acid phosphatase: isolation of genes for two novel phytases from fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology 143:245–252

Mullaney EJ, Ullah AH (2003) The term phytase comprises several different classes of enzymes. Biochem Biophys Res Commun 312:179–184

Mullaney EJ, Daly CB, Ullah AHJ (2000) Advances in phytase research. Adv Appl Microbiol 47:157–199

Murugesan GS, Sathishkumar M, Swaminathan K (2005) Supplementation of waste tea fungal biomass as a dietary ingredient for broiler chicks. Bioresour Technol 96:1443–1448

Nagashima T, Tange T, Anazawa H (1999) Dephosphorylation of phytate by using *Aspergillus niger* phytase with a high affinity for phytate. Appl Environ Microbiol 65(10):4682–4684

Nakamura Y, Fukuhara H, Sano K (2000) Secreted phytase activities of yeasts. Biosci Biotechnol Biochem 64:841–844

Nampoothiri KM, Tomes GJ, Roopesh K, Szakacs G, Nagy V, Soccol CR, Pandey A (2004) Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. Appl Biochem Biotechnol 118:205–214

Nayini NR, Markakis P (1984) The phytase of yeast. Lebensmittel-Wissenchaftund Technologie 17:24–26

Nelson T, Shieh TR, Wodzinski RJ, Ware JH (1971) Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J Nutr 101:1289–1293

Pandey P, Selvakumar P, Soccol CR et al (1999) Solid state fermentation for the production of industrial enzymes. Curr Sci 77:149–162

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001) Production, purification and properties of microbial phytases. Bioresour Technol 77:203–214

Pasamontes L, Haiker M, Henriquez-Huecas M, Mitchell DB, van Loon APGM (1997a) Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. Biochim Biophys Acta 1353:217–223

Pasamontes L, Haiker M, Wyss M, Tessier M, van Loon APGM (1997b) Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. Appl Environ Microbiol 63:1696–1700

Pillai UP, Manoharan V, Lisle A, Li X, Bryden W (2009) Phytase supplemented poultry diets affect soluble phosphorus and nitrogen in manure and manure-amended soil. J Environ Qual 38:1700–1708

Powar VK, JagannathanV (1967) Phytase from *Bacillus subtilis*. Indian J biochem 4(3):184–185

Powar VK, Jagannathan V (1982) Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J Bacteriol 151:1102–1108

Quan CS, Zhang LH, Wang YJ, Ohta YY (2001) Production of phytase in a low phosphate medium by a novel yeast *Candida krusei*. J Biosci Bioeng 92(2):154–160

Quan CS, Fan SD, Zhang LH, Wang YJ, Ohta Y (2002) Purification and properties of a phytase from *Candida krusei* WZ-001. J Biosci Bioeng 94(5):419–425

Raghavendra P, Halami PM (2009) Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. Int J Food Microbiol 133(1–2):129–134

Ramachandran S, Krishnan R, Nampoothiri KM, Szackacs G, Pandey A (2005) Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oil cakes as substrates. Process Biochem 40(5):1749–1754

Rao DE, Rao KV, Reddy TP, Reddy VD (2009) Molecular characterization, physicochemical properties, known and potential applications of phytases: an overview. Crit Rev Biotechnol 29(2):182–198

Reddy NR, Sathe SK, Salunkhe DK (1982) Phytases in legumes and cereals. Adv Food Res 82:1–92

Revy PS, Jondreville C, Dourmad JY, Nys Y (2006) Assessment of dietary zinc requirement of weaned piglets fed diets with or without microbial phytase. J Anim Physiol Anim Nutr 90(1–2):50–59

Richard LC, Stephen RC, John CP, Patricia AS, Craig A (1998) Phosphorus loading reductions needed to control blue-green algal blooms in Lake Mendota. Can J Fish Aquat Sci 55(5):1169–1178

Robinson EH, Li MH, Manning BB (2002) Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (*Oncorhynchus tshawytscha*). Comparison of microbial phytase and dicalcium phosphate for growth and bone mineralization of pond-raised channel catfish, *Ictalurus punctatus*. J Appl Aquacult 12:81–88

Rodehutscord M, Pfeffer E (1995) Effects of supplemental microbial phytase on phosphorus digestibility and utilization in rainbow trout (*Oncorhynchus mykiss*). Water Sci Technol 31:143–147

Rodriguez E, Porres JM, Han Y, Lei XG (1999) Different sensitivity of recombinant *Aspergillus niger* phytase (r-PhyA) and *Escherichia coli* pH 2.5 acid phosphatase (r-ppA) to trypsin and pepsin *in vitro*. Arch Biochem Biophys 365:262–267

Rodriguez E, Mullaney EJ, Lei XG (2000) Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. Biochem Biophys Res Commun 268:373–378

Sajjadi M, Carter CG (2004) Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in atlantic salmon (*Salmo salar*, L.). Aquacult Nutr 10:135–142

Sandberg AS, Larsen T, Sandstorm B (1993) High dietary calcium level decreases phytate degradation in pigs fed a rapeseed diet. J Nutr 123:559–566

Sano L, Fukuhara H, Nakamura Y (1999) Phytase of the yeast *Arxula adeninivorans*. Biotechnol Lett 21:33–38

Schafer A, Koppe WM, Meyer-Burgodorff KH, Gunther KD (1995) Effects of a microbial phytase on the utilization of native phosphorus by carp in a diet based on a soyabean meal. Water Sci Technol 31:149–155

Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P (1992) Purification and properties of phytase from *Schwanniomyces castellii*. J Ferment Bioeng 74:7–11

Selle PH, Ravindran R (2007) Microbial phytase in piggery nutrition. Anim Feed Sci Technol 135:1–41

Shieh TR, Ware JH (1968) Survey of microorganism for the production of extracellular phytase. Appl Microbiol 16:1348–1351

Shieh TR, Wodzinski RJ, Ware JH (1969) Regulation of the formation of acid phosphatase by inorganic phosphate in *Aspergillus ficuum*. J Bacteriol 100:1161–1165

Shimizu M (1992) Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. Biosci Biotechnol Biochem 56:1266–1269

Shimizu M (1993) Purification and characterization of phytase and acid phosphatase produced by *Aspergillus oryzae* K1. Biosci Biotechnol Biochem 57:1364–1365

Singh B, Satyanarayana T (2006a) Phytase production by thermophilic mold *Sporotrichum thermophile* in solid-state fermentation and its application in dephytinization of sesame oil cake. Appl Biochem Biotechnol 133:239–250

Singh B, Satyanarayana T (2006b) A marked enhancement in phytase production by a thermophilic mould *Sporotrichum thermophile* using statistical designs in a cost-effective cane molasses medium. J Appl Microbiol 101:344–352

Singh B, Satyanarayana T (2008a) Improved phytase production by a thermophilic mould *Sporotrichum thermophile* in submerged fermentation due to statistical optimization. Bioresour Technol 99:824–830

Singh B, Satyanarayana T (2008b) Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its potential applications. Bioresour Technol 99:2824–2830

Singh B, Satyanarayana T (2008c) Phytase production by *Sporotrichum thermophile* in a cost-effective cane molasses medium in submerged fermentation and its application in bread. J Appl Microbiol 105:1858–1865

Singh B, Satyanarayana T (2009) Characterization of a HAP-phytase from a thermophilic mould *Sporotrichum thermophile*. Bioresour Technol 100:2046–2051

Singh B, Satyanarayana T (2010) Plant growth promotion by an extracellular HAP-phytase of a thermophilic mold *Sporotrichum thermophile*. Bioresour Technol 100:2046–2051

Singh B, Satyanarayana T (2011a) Phytases from thermophilic molds: their production, characteristics and multifarious applications. Process Biochem 46(7):1391–1398

Singh B, Satyanarayana T (2011b) Microbial phytases in phosphorus acquisition and plant growth promotion. Physiol Mol Biol Plants 17(2):93–103

Singh B, Kaur P, Satyanarayana T (2006) Fungal phytases for improving the nutritional status of foods and combating environmental phosphorus pollution. In: Chauhan AK, Verma A (eds) Microbes: health and environment. IK International Publishers, New Delhi, pp 289–326

Singh B, Kunze G, Satyanarayana T (2011) Developments in biochemical aspects and biotechnological applications of microbial phytases. Biotechnol Mol Biol Rev 6(3):69–87

Siren M (1986a) Stabilized pharmaceutical and biological material composition. Patent SE 003, 165

Siren M (1986b) New myo-inositol triphosphoric acid isomer. Patent SW 052, 950

Sreeramulu G, Srinivasa DS, Nand K, Joseph R (1996) *Lactobacillus amylovorus* as a phytase producer in submerged culture. Lett Appl Microbiol 23:385–388

Stahl CH, Han YM, Roneker KR, House WA, Lei XG (1999) Phytase improves iron bioavailability for hemoglobin synthesis in young pigs. J Anim Sci 77:2135–2142

Stahl CH, Wilson DB, Lei XG (2003) Comparison of extracellular *Escherichia coli* AppA phytases expressed in *Streptomyces lividans* and *Pichia pastoris*. Biotechnol Lett 25(10):827–831

Sunita K, Kim YO, Lee JK, Oh TK (2000) Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. Biochem Eng J 5:51–56

Suzuki U, Yoshimura K, Takaishi M (1907) Ueber ein Enzym "Phytase" das "Anhydro-oxy-methylen diphosphorsaure" Spaltet. Tokyo Imperal Univ Coll Agric Bull 7:503–512

Tambe SM, Kakli SG, Kelkar SM, Parekh LJ (1994) Two distinct molecular forms of phytase from *Klebsiella aerogenes*; evidence for unusually small active enzyme peptide. J Ferment Bioeng 77:23–27

Tatala S, Svanberg U, Mduma B (1998) Low dietary iron availability is a major cause of anemia: a nutrition survey in the Lindi District of Tanzania. Am J Clin Nutr 68:1171–1178

Tyagi PK, Verma SVS (1998) Phytate phosphorus content of some common poultry feed stuffs. Indian J Poult Sci 33:86–88

Tye AJ, Siu FK, Leung TY, Lim BL (2002) Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. Appl Microbiol Biotechnol 59(2–3):190–197

Ullah AHJ, Sethumadhavan K, Mullaney EJ, Ziegelhoffer T, Austin-Phillips S (2002) Cloned and expressed fungal *phyA* gene in alfalfa produces a stable phytase. Biochem Biophys Res Commun 290:1343–1348

Usmani N, Jafri AK (2002) Influence of dietry phytic acid on growth, conversion efficiency and carcass composition of mrigal *Cirrhinus mrigala* (hamilton) fry. J World Aquacult Soc 33:199–204

Van Weerd JH, Khalaf KHA, Aartsen FJ, Tijssen PAT (1999) Balance trials with African catfish *Clarias gariepinus* fed phytase-treated soybean meal-based diets. Aquacult Nutr 5:135–142

Vats P, Banerjee UC (2002) Studies on the production of phytase by a newly isolated sp. of A*spergillus niger* van Teigham obtained from rotten wood-logs. Process Biochem 38:211–217

Vats P, Banerjee UC (2004) Production studies and catalytic properties of phytases (*myo*-inositolhexakisphosphate phosphohydrolases): an overview. Enzyme Microb Technol 35:3–14

Vats P, Banerjee UC (2005) Biochemical characterisation of extracellular phytase (*myo*-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem. J Ind Microbiol Biotechnol 32:141–147

Vats P, Bhushan B, Banerjee UC (2009) Studies on the dephosphorylation of phytic acid in live stock and feed using phytase from *Aspergillus niger* van Teighem. Bioresour Technol 100:287–291

Vielma J, Ruohonen K, Gabaudan J, Vogel K (2004) Top-spraying soybean meal-based diets with phytase improves protein and mineral digestibilities but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (walbaum). Aquacult Res 35:955–964

Vohra A, Satyanarayana T (2001) Phytase production by the yeast *Pichia anomala*. Biotechnol Lett 23:551–554

Vohra A, Satyanarayana T (2002) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem 37:999–1004

Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23:29–60

Vohra A, Satyanarayana T (2004) A cost-effective cane molasses medium for enhanced cell-bound phytase production by *Pichia anomala*. J Appl Microbiol 97:471–476

Vohra A, Rastogi SK, Satyanarayana T (2006) Amelioration in growth and phosphate assimilation of poultry birds using cell-bound phytase of *Pichia anomala*. World J Microbiol Biotechnol 22:553–558

Vohra A, Kaur P, Satyanarayana T (2011) Production, characteristics and applications of the cell-bound phytase of *Pichia anomala*. Antonie Van Leeuwenhoek 99:51–55

Volfova O, Dvorakova J, Hanzlikova A, Jandera A (1994) Phytase *from Aspergillus niger*. Folia Microbiol (Praha) 39(6):481–484

Wang HL, Savain W, Hesseltine CW (1980) Phytase of molds used in oriental food fermentation. J Food Sci 45:1261–1266

Wang Y, Gao X, Su Q, Wu W, An L (2007) Cloning, expression and enzyme characterization of an acid heat stable phytse from *Aspergillus fumigatus* WY-2. Curr Microbiol 55(1):65–70

Wodzinski RJ, Ullah AHJ (1996) Phytase. Adv Appl Microbiol 42:263–301

Wyss M, Brugger R, Kronenberger A, Remy R, Fimbel R, Oesterhelt O (1999a) Biochemical characterization of fungal phytases (*myo*inositolhexakisphosphate- phosphohydrolases): catalytic properties. Appl Environ Microbiol 65:367–373

Wyss M, Pasamontes L, Friedlein A, Remy R, Tessier M, Kronenberger A (1999b) Biophysical characterization of fungal phytases (*myo*-inositolhexakisphosphate-phosphohydrolases): molecular size, glycosylation pattern and engineering of proteolytic resistance. Appl Environ Microbiol 65:359–366

Xiao K, Harrison MJ, Wang ZY (2005) Transgenic expression of a novel *M. truncatula* phytase gene results in improved acquisition of organic phosphorus by *Arabidopsis*. Planta 222(1):27–36

Yadav RS, Tarafdar JC (2003) Phytase and phosphatase producing fungi in arid and semi-arid soils and their efficiency in hydrolyzing different organic P compounds. Soil Biol Biochem 35(6):745–751

Yan W, Reigh RC (2002) Effects of fungal phytase on utilization of dietary proteins and minerals and dephosphorylation of phytic acid in the alimentary tract of channel catfish, *Ictalurus punctatus* fed an all plant-protein diet. J World Aquacult Soc 33:10–22

Yanke LJ, Bae HD, Selinger LB, Cheng KJ (1998) Phytase activity of anaerobic ruminal bacteria. Microbiology 144(Pt 6):1565–1573

Yanke LJ, Selinger LB, Cheng KJ (1999) Phytase activity of *Selenomonas ruminantium*: a preliminary characterization. Lett Appl Microbiol 29:20–15

Yi Z, Kornegay ET, Ravindran V, Denbow DM (1996) Improving phytate phosphorus availability in corn and soybean meal for broilers using microbial phytase and calculation of phosphorus equivalency values for phytase. Poult Sci 75:240–249

Yip W, Wang L, Cheng C, Wu W, Lung S, Lim BL (2003) The introduction of a phytase gene from *Bacillus subtilis* improved the growth performance of transgenic tobacco. Biochem Biophys Res Commun 310:1148–1154

Yoon SJ, Choi YJ, Min K, Cho KK, Kim JW, Lee SC (1996) Isolation and identification of phytase producing bacterium, *Enterobacter* sp. 4 and enzymatic properties of phytase enzyme. Enzyme Microb Technol 18:449–454

Zhang GQ, Dong XF, Wang ZH, Zhang Q, Wang HX, Tong JM (2010) Purification, characterization, and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. Bioresour Technol 101:4125–4131

Bioremediation Concepts for Treatment of Distillery Effluent

14

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Abstract

Distillery spent wash from alcohol manufacturing units such as breweries is highly polluted with organic components and is amongst the most polluting agroindustrial waste. Despite stringent standards imposed on effluent quality, untreated or partially treated effluents very often find access to watercourses. The distillery wastewater poses a serious threat to water quality in several regions around the globe. Lowering of pH values of the stream, increasing organic load, depletion of dissolved oxygen content, destruction of aquatic life and odour are some of the major pollution problems due to distillery wastewaters. Increasing number of biological clean-up technologies is being described, and novel bioremediation approaches for treatment of distillery spent wash are being worked out. Potential microbial processes (anaerobic and aerobic) for developing feasible remediation technologies to combat environmental pollution due to distillery spent wash are in progress.

Keywords

Anaerobic reactors • Bioremediation • Distillery spent wash • Melanoidins • Microorganisms

Introduction

Alcohol distilleries are one of the most polluting industries generating an average of 8–15 L of effluent, which is termed as distillery spent wash per litre of alcohol produced (Saha et al. 2005). Several important industrial applications of alcohol such as in pharmaceuticals, food and perfumery as well as its use as an alternative fuel have promoted extensive growth of alcohol distilleries all over the world, and this industrial growth has also led to generation of massive quantities of the effluent. There are 319 distilleries in India alone producing 3.25 billion litres of alcohol and generating 40.4 billion litres of wastewaters annually (Pant and Adholeya 2007a).

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Alcohol production in distilleries consists of four main steps, i.e. feed preparation, fermentation, distillation and packaging (Satyawali and Balakrishnan 2007). Ethanol can be prepared from various biomass materials, but the potential for use of these materials as feedstock for industrial scale ethanol production depends on their cost, availability, their carbohydrate contents and the ease by which they can be converted to alcohol (Ogbonna 2004). Nearly 61% of world ethanol production is from sugar crops (Berg 2004).

Most Indian distilleries exclusively use cane molasses as raw material for fermentation (Handa and Seth 1990). Molasses is suitably diluted in order to have desired sucrose level in it. It is then supplemented with assimilable nitrogen source like ammonium sulphate or urea. It is also supplemented with phosphate if necessary. The pH of the fermentation broth is adjusted below five using sulphuric acid. Fermentation is carried out for about 50 h by using 5% active culture of *Saccharomyces cerevisiae*. Ethanol accumulates to 8–10% in the fermented mash. The fermented mash is then distilled, fractionated and rectified after the removal of yeast sludge (Pathade 2003). Apart from yeasts, a bacterial strain, *Zymomonas mobilis*, has been demonstrated as a potential candidate for ethanol production (Chandraraj and Gunasekaran 2004). The residue of the fermented mash which comes out as liquid waste is termed as spent wash (Pathade 2003; Singh et al. 2004; Nandy et al. 2002).

The complex, caramelised and cumbersome agroindustrial wastewater generated from distillation of fermenter mash is in the temperature range of 70–80°C, deep brown in colour and acidic in nature (low pH) with high concentration of organic materials, and solids. However, the pollution load of the distillery effluent depends on the quality of molasses, unit operations for processing of molasses and process recovery of alcohols (Pandey et al. 2003).

With government policies on pollution control becoming more stringent, distillery industries have been forced to look at more effective treatment technologies, which would not only be beneficial to environment, but also reduce capital costs. Consequently, extensive treatment is required before the treated wastewater can meet the stipulated environmental demands.

This chapter presents the state of art and the experiences gained so far on different technologies and implementation strategies for promotion of treatment of distillery spent wash.

Pollution Potential of Distillery Spent Wash

The production and the characteristics of the spent wash are highly variable and dependent on the raw material used and various aspects of the ethanol production process (Satyawali and Balakrishnan 2007; Pant and Adholeya 2007a). Wash water used to clean the fermenters, cooling water blowdown and broiler water blowdown further contributes to its variability (Pant and Adholeya 2007a). Distillery spent wash has very high BOD (biological oxygen demand), COD (chemical oxygen demand) and high amounts of nitrogen, potassium, phosphates, calcium, sulphates and other solids (Table 14.1). Its recalcitrant nature is due to presence of brown polymers melanoidins, which are formed by Maillard amino carbonyl reaction. These compounds have antioxidant properties, which render them toxic to many microorganisms typically present in wastewater treatment processes (Kumar et al. 1997). The defiance of melanoidins to degradation is apparent from the fact that these compounds escape various stages of wastewater treatment plants and finally enter into the environment. Apart from melanoidins, the other recalcitrant compounds present in the waste are caramel, variety of sugar decomposition products, anthocyanins and tannins and other xenobiotic compounds formed during yeast growth and processing of alcohol (Pandey et al. 2003). Spent wash disposal into the environment is hazardous and has high pollution potential. Its high COD, total nitrogen and total phosphate means that its disposal into natural water bodies may result in eutrophication (Kumar et al. 1997). The highly coloured components of the spent wash lead to a reduction of sunlight penetration in rivers, lakes or lagoons which in turn decreases

Table 14.1 Characteristics of raw and anaerobically treated distillery effluent[a]

Parameters	Values of distillery spent wash	Values of anaerobically treated effluent
pH	3.0–4.5	7.5–8
BOD_5 (mgL^{-1})	50,000–60,000	8,000–10,000
COD (mgL^{-1})	1,10,000–1,90,000	45,000–52,000
Total solid (TS) (mgL^{-1})	1,10,000–1,90,000	70,000–75,000
Total volatile solid (TVS) (mgL^{-1})	80,000–1,20,000	68,000–70,000
Total suspended solid (TSS) (mgL^{-1})	13,000–15,000	38,000–42,000
Total dissolved solids (TDS) (mgL^{-1})	90, 000–1,50,000	30,000–32,000
Chlorides (mgL^{-1})	8,000–8,500	7,000–9,000
Phenols (mgL^{-1})	8,000–10,000	7,000–8,000
Sulphate (mgL^{-1})	7,500–9,000	3,000–5,000
Phosphate (mgL^{-1})	2,500–2,700	1,500–1,700
Total nitrogen (mgL^{-1})	5,000–7,000	4,000–4,200

[a]Adapted from Acharya et al. (2008) and Mohana et al. (2007)

both photosynthetic activity and dissolved oxygen concentration causing harm to aquatic life. Saxena and Chauhan (2003) investigated the effect of influence of distillery effluent on oxygen consumption in freshwater fish *Labeo rohita* and concluded that presence of inorganic and organic salts in the effluent interfered with the respiration in the fish by coagulating gill mucous and caused asphyxiation, inhibition of mitochondrial enzyme and thus decreased dissolved oxygen consumption. Matkar and Gangotri (2003) observed concentration-dependent toxicity of distillery effluent on the freshwater crab *Barytelphusa guerini.* Impact of distillery effluent on carbohydrate metabolism of *Cyprinus carpio*, a freshwater fish, was studied by Ramakritinan et al. (2005). Stress due to distillery effluent caused defunct respiratory processes in the fish resulting in anaerobiosis at organ level during sublethal intoxication.

Disposal on land is equally hazardous causing a reduction in soil alkalinity and manganese availability, inhibition of seed germination and ruin of vegetation (Kumar et al. 1997). In a study carried out by Dhembare and Amin (2002), indices indicating soil quality like sodium absorption ratio (SAR), soluble sodium percentage (SSP) and Kelly's ratio were adversely affected in the soil which was amended with distillery effluent. Constant disposal/irrigation of the soil with effluent led to deleterious effect on the soil properties. Use of distillery effluent in agriculture is controversial as researchers have reported its beneficial as well as its detrimental effect on crop yield and soil properties (Pathak et al. 1999).

Treatment Technologies

A number of technologies have been explored for the reduction of pollution load of distillery effluent. Various physico-chemical methods have been put into practice for treatment of distillery effluent such as adsorption, coagulation–flocculation, and oxidation processes such as Fenton's oxidation, ozonation, electrochemical oxidation using various electrodes and electrolytes, nanofiltration, reverse osmosis, ultrasound and different combinations of these methods. Majority of these methods remove colour by either concentrating the colour into sludge or by partial or complete breakdown of the colour molecules. Nevertheless, the disadvantages associated with these methods are excess usage of chemicals, sludge generation with subsequent disposal problems, high installation as well as operational costs and sensitivity to variable wastewater input.

Despite the existence of a variety of chemical and physical treatment processes, bioremediation of distillery effluent is seen as an attractive solution due

to its reputation as a low-cost, environment-friendly and socioeconomically acceptable technology. The level of technology used and its effectiveness depend on the distillery and ultimately by national and international legislations.

Biological treatment of distillery spent wash is either aerobic or anaerobic but in most cases a combination of both. A typical COD/BOD ratio of 1.8-1.9 indicates the suitability of the effluent for biological treatment (Singh et al. 2004). Aerobic treatment of high organic load such as molasses is associated with operational difficulties of sludge bulking, inability of the system to treat high BOD or COD loads economically, relatively high biomass production and high cost in terms of energy (Jimnez et al. 2003). Moreover, a BOD:N:P ratio of 100:2.4:0.3 suggests that anaerobic treatment methods will be more effective than aerobic treatment methods for reducing the pollution potential of distillery effluent.

Anaerobic treatment of distillery effluent is an accepted practice, and various high-rate reactor designs have been tried at pilot and full-scale operation (Lata et al. 2002).

Anaerobic Treatment

Anaerobic digestion is viewed as a complex ecosystem in which physiologically diverse groups of microorganisms operate and interact with each other in a symbiotic, synergistic, competitive and antagonistic association in the process generating gas, which is mainly methane and carbon dioxide. The overall biochemistry of the process involves defined carbon and electron flow pathways for the metabolism of diverse organic substrates into simple carbon products. The use of C^{14} tracer studies and the isolation of prevalent species and bacterial characterisation studies have resulted in a good quantitative picture of carbon flow in biomethanation. The anaerobic microbial food chain consists of mainly three functionally different groups of organism, namely, hydrolytic fermentative, syntrophic acetogenic and methanogenic bacteria (Jain et al. 1990).

Methanogens posses very limited metabolic repertoire, using only acetate or C_1 compounds (H_2 and CO_2, formate, methanol, methylamines or CO), with methane being end product of the reaction (Fig. 14.1). Of the methanogenic genera, *Methanosarcina* sp. and *Methanosaeta* sp. form methane by the acetoclastic reaction. Fast-growing *Methanosarcina* sp. is predominant in high-rate, shorter retention digesters where in acetate concentration is higher. *Methanosaeta* sp. is predominant in low-rate slow turn over digesters. Both carbon dioxide-reducing and acetoclastic methanogens play an important role in maintaining stability of the digester. The failure in an anaerobic digester can occur if carbon dioxide-reducing methanogens fail to keep pace with hydrogen production (Nagamani and Ramasamy 1999).

Wastewater treatment using anaerobic process is a very promising re-emerging technology which presents extremely interesting advantages compared to classical aerobic treatment: it has high capacity of degrading concentrated and resilient substances, produces very little sludge and requires little energy, and it can become profitable by cogenerating useful biogas (Mailleret et al. 2003). However, these processes have been sensitive to organic shock loadings, have low pH and show slow growth rates of anaerobic microbes which lead to longer hydraulic retention times (HRT). This often results in poor performance of in conventional mixed reactors. In order to solve these problems, several high-rate configurations have been developed for treating soluble wastewater at relatively shorter HRTs (Patel and Madamwar 2000).

Realising the importance of minimum cell residence time (MCRT) as a process control factor, a number of anaerobic processes are available today depending in the way microbial biomass is retained in the reactor. Attempts to overcome the disadvantages of treating different industrial waste have led to development of various kinds of anaerobic processes (Joshi 1999).

Single-Phasic and Biphasic Anaerobic Systems

Anaerobic systems can be operated as single-phase or two-phase systems. Single-phase systems involve only one reactor for the

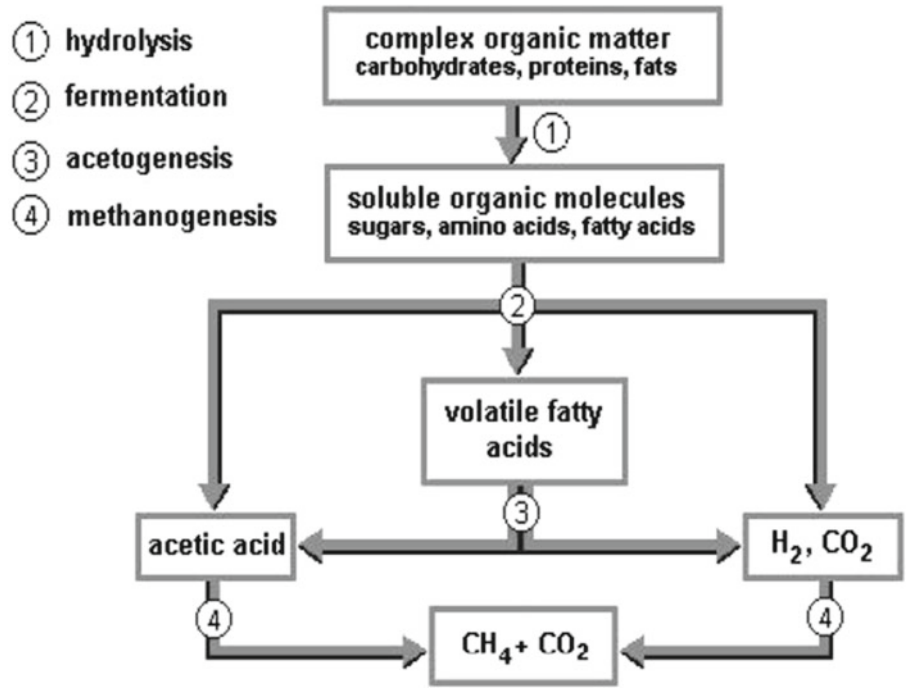

Fig. 14.1 Metabolic route for conversion of organic matter to the methanogenic substrates (acetate carbon dioxide and hydrogen) and finally to methane and carbon dioxide

microorganisms to digest the organic matter, whereas two-phase systems separate the hydrolysis and acidogenic and methanogenic organisms into two separate reactors. A biphasic system is capable of optimising the fermentation steps of each stage in separate fermenters with a result that overall biphasic process efficiency and kinetics are higher than those of conventional single-stage processes in which all primary and secondary organisms and associated fermentations are conducted under the same identical environmental conditions of a single-stage digester. In primary phase fermentation step, the fermentation end products are formate, acetate and C_3 and higher volatile fatty acids, lactate, ethanol, carbon dioxide and hydrogen. It is basically acid fermentation phase. The secondary phase is acetotrophic methane fermentation where the end products are methane and carbon dioxide (Ghosh 1990). Biomethanation using biphasic system is most appropriate treatment method for high-strength wastewater because of its reported advantages, namely, possibility of maintaining optimal conditions for buffering of imbalances between organic acid production and consumption, stable performance and higher methane concentration in the biogas produced (Seth et al. 1995).

Anaerobic Lagooning

Anaerobic lagoons are the simplest choice for anaerobic treatment of distillery waste. Rao (1972) studied the applicability of anaerobic lagoon treatment of distillery waste in two pilot-scale lagoons in series to offer overall BOD removals ranging from 82 to 92%.

However, the lagoon systems are seldom operational, souring being a frequent phenomenon. The groundwater contamination cannot be prevented as these lagoons are generally unlined. They require large area to treat large volumes of waste, and they also lead to odour nuisance (Handa and Seth 1990; Singh et al. 2004).

Conventional Anaerobic Reactors

The conventional digesters such as continuous stirred tank reactors (CSTR) are the simplest form

of closed reactors with supervision of gas collection. Treatment of distillery effluent in CSTR has been reported in single as well as biphasic operations, resulting in 80–90% COD reduction in 10–15 days (Pathade 2003). The hydraulic retention time (HRT) in CSTR-type reactor is determined by the specific growth rate of the slowest growing microorganism in the system. This generally means that very high HRT values are required to achieve an acceptable level of degradation. The high HRT values make the CSTR concept less feasible and unattractive for treatment of the wastewaters (Kleerebezem and Macarie 2003).

High-Rate Reactors

Anaerobic Fixed Film Reactors

In fixed film reactors, the reactor has a support structure (media) for biomass attachment. Fixed film offers the advantages of simplicity of construction, elimination of mechanical mixing, better stability even at higher loading rates and capability to withstand larger toxic shock loads. The reactors can recover very quickly after a period of starvation (Rajeshwari et al. 2000). Amongst numerous anaerobic reactors developed for biomethanation, anaerobic fixed film reactors (AFFR) have emerged as the most popular one compared to other reactors due to availability of large biomass in the reactor (Patel and Madamwar 2002). The colonisation process proceeds in three consecutive phases: lag phase (primary cellular attachment), biofilm production (bacterial accumulation with production of biopolymer matrix) and steady-state establishment (establishment of a mature biofilm) (Michaud et al. 2002). The nature of the media used for biofilm attachment has a significant effect on reactor performance. A wide variety of materials have been used as non-porous support media at laboratory and pilot scale, including glass bead, red drain clay, sand and a number of different plastics and porous materials such as needle-punched polyesters, polyurethane foam and sintered glass (Perez et al. 1997), waste tyre rubber (Borja et al. 1996), poly(acrylonitrile–acrylamide) (Lalov et al. 2001) and corrugated plastic (Perez-Garcia et al. 2005).

Jhung and Choi (1995) performed a comparative study of UASB (Upflow Anaerobic Sludge Blanket) and anaerobic fixed film reactors for treatment of molasses waste. The fixed film reactor was fabricated with a total volume of 5.4 L, filled with Koch plastic media having a porosity of 93–95%, a diameter of 1.6 cm and a specific area of 345 m^2m^{-3}, and the total volume of the UASB reactor was 4.4 L. The fixed film reactor was found to be more efficient than the UASB reactor as it could be operated at higher OLR (organic loading rates) (19 kg COD $m^{-3}d^{-1}$) than UASB (12.5 kg COD m^{-3} d^{-1}), and it produced higher COD removal efficiencies at OLR of 10 kg COD m^{-3} d^{-1}. The better performance of the fixed film reactor was attributed its ability to retain higher biomass even at higher OLR. Seth et al. (1995) carried out comparative studies on the performance of two different support material, namely, granular activated carbon (GAC) and clay brick granules (CBG) on biomethanation of distillery spent wash in a biphasic fixed film reactor. The maximum OLR achieved with GAC was 21.3 kg COD m^{-3} d^{-1}, corresponding to a HRT of 4 d with COD and TVA reductions of 67 and 82%, respectively, whereas OLR achieved with CBG was 22 kg COD m^{-3} d^{-1}, corresponding to a HRT of 3d with COD and TVA reductions of 71.8 and 88.5%, respectively. The better performance of CBG over GAC as support material was attributed to its better support characteristics which were confirmed by SEM analysis.

Thermophilic stability of the fixed film reactors was investigated by Perez et al. (1997) using anaerobic fixed film reactor packed with porous sintered glass support. This carrier was termed as SIRAN and was produced by sintering of a mixture of glass and salt powder. The resulting sponge had a well-defined pore size distribution (double pore structure) which resulted in 80% COD reduction at a COD loading rate of 3.81 kg COD $m^{-3}d^{-1}$ within 75 days. Thus the study revealed that under thermophilic anaerobic conditions, the support material enabled fast attachment of the microorganisms resulting in short start-up and stable operation. In another study, Perez-Garcia et al. (2005) studied the influent pH conditions in fixed film reactors for

anaerobic thermophilic treatment of wine distillery wastewaters. The results obtained showed that the pH of the influent influences the performance of the biodegradation process and the depurative efficiency is higher for the operation with alkaline influent. The operation with acid influent allowed the reactor to operate at organic loading rates (OLR) around 5.6 kg COD $m^{-3}d^{-1}$ (hydraulic retention time: 1.5 days), maintaining total chemical oxygen demand (COD) removal of 77.2%; the operation with alkaline influent allowed total COD removal of 76.8% working at OLR around 10.5 kg COD m^{-3} d^{-1}. The greatest efficiency of substrate removal was 87.5% for OLR 3.2 kg COD $m^{-3}d^{-1}$ and hydraulic retention time of 4 days operating with alkaline influent. Therefore, the operation with alkaline influent implicates senior levels of purifying efficiency for similar organic load rate. Acharya et al. (2008) performed a comparative study of low-cost packing materials for the treatment of distillery spent wash using anaerobic fixed film reactors. They found coconut coir as the best supporting material, as the system supported the treatment at very high organic loading rate of 31 kg COD $m^{-3}d^{-1}$ with 50% COD reduction. Charcoal and nylon fibres were other packing materials used in the study. Charcoal was able to retain the active biomass at the OLR of 15.5 kg COD $m^{-3}d^{-1}$ resulting in more than 60% COD reduction, but nylon fibres failed to support the biofilm development even at higher HRT and lower OLR.

Upflow Anaerobic Sludge Blanket (UASB) Reactors

In the recent years, the UASB process has been successfully used for the treatment of various types of wastewaters (Lettinga and Hulshoff Pol 1991). UASB reactor systems belong to the category of high-rate anaerobic wastewater treatment, and hence it is one of the most popular and extensively used for treatment of distillery wastewaters globally. The success of UASB depends on the formation of active and settleable granules (Fang et al. 1994). These granules consist of aggregation of anaerobic bacteria self-immobilised into compact forms. This enhances the settleability of biomass and leads to an effective retention of bacteria in the reactor (Akunna and Clark 2000). Particularly attractive features of the UASB reactor design include its independence from mechanical mixing of digester contents, recycling of sludge biomass (Kalyuzhnyi et al. 1997) and ability to cope up with perturbances caused by high loading rates and temperature fluctuations (Sharma and Singh 2000). The UASB technology is well suited for high-strength distillery wastewaters only when the process has been successfully started up and is in stable operation. To achieve successful start-ups, the reactors must be operated at a low loading rate of between 4 and 8 kg COD $m^{-3}d^{-1}$, and the COD removal efficiency must be monitored carefully. Once the COD removal efficiencies are above 90%, then the loading rate can be increased (Wolmarans and Villiers 2002). Malt whisky distillery pot ale, a liquid waste product from the malt whisky industry, treated in a laboratory-scale UASB reactor (Goodwin and Stuart 1994) indicated the importance of dilution and pH control in attaining a high COD reduction. There is normally a rise in the pH due to ammonia production during the process of digestion. The maximum loading rate for a stable operation was 15 kg COD $m^{-3}d^{-1}$ at a retention time of 2.1 days. Florencio et al. (1997) investigated the environmental factors that are of importance in the predominance of methylotrophic methanogens over acetogens in a natural mixed culture during anaerobic treatment in UASB reactors. An increased growth rate of the methanogens at higher temperatures makes the thermophilic anaerobic digestion process a suitable alternative to mesophilic digestion (Rajeshwari et al. 2000). Harada et al. (1996) investigated the feasibility of UASB reactors at thermophilic temperatures. A 140-L UASB reactor was studied for a period of 430 days. Organic loading rate was applied up to 28 kg COD $m^{-3}d^{-1}$ by reducing hydraulic retention time at a fixed influent concentration of 10 kg COD $m^{-3}d^{-1}$. COD removal was about 67%, while BOD removal was more satisfactory (>80%). Successful operation of the UASB reactors treating distillery waste at psychrophilic temperatures (4–10°C) was studied by operating one- and two-stage UASB reactors. The organic

loading rate varied from 4.7 to 1.3 g COD at hydraulic retention times of 6–7 days for one-stage reactor and 2 days for the two-stage reactor. The average total COD removal for vinasses wastewaters was 60% in the one-stage reactor and 70% in the two-stage reactors. In situ determinations of kinetic sludge characteristics (apparent V_m and K_m) revealed the existence of substantial mass transfer limitations for the soluble substrates inside the reactor sludge bed. Therefore, application of higher recycle ratios is essential for enhancement of UASB pretreatment under psychrophilic conditions (Kalyuzhnyi et al. 2001). The conventional UASB reactors concept showed severe limitations mainly owing to problems related to mass transfer resistance or the appearance of concentration gradients inside the systems (van Lier et al. 2001).

Some other disadvantages of the process are primary start-up which requires several weeks; granulation process is difficult to control and depends on a large number of parameters, and as organic load increases, a proper monitoring of the process needs to be done especially to maintain requisite alkalinity to counter excessive acid accumulation (Ghangrekar et al. 2003). In last decades, the system-specific parameters of UASB reactors have been modified to increase the loading potentials and/or to widen the applicability of anaerobic reactor systems for various types of wastewaters (van Lier et al. 2001). By making use of the high settleability of the methanogenic sludge granules (40–60 mh^{-1}), expanded granular sludge bed (EGSB) systems were developed, which are operated at upflow velocities exceeding 8 mh^{-1}, brought about by an increased height/diameter ratio and external circulation pump. In contrast to the conventional UASB reactor, the EGSB systems are not equipped with an internal settler but with an advanced gas–liquid–solid separation device (van Lier et al. 2001). As a result of the excellent contact between wastewaters and sludge, these systems can handle higher organic loading rates, while they are also less sensitive for negative effects of suspended solids present in the wastewaters than conventional UASB systems (Lettinga 1990). Anaerobic treatment of low-strength brewery wastewater, with influent total chemical oxygen demand (CODin) concentrations ranging from 550 to 825 mg/L, was investigated in a pilot-scale 225.5-L expanded granular sludge bed (EGSB) reactor. At 20°C, COD removal efficiencies exceeding 80% were obtained at an OLR up to 12.6 kg COD $m^{-3}d^{-1}$, with CODin between 630 and 715 mgL^{-1}. The values of HRT and liquid upflow velocity applied were 2.1–1.2 h and 4.4–7.2 mh^{-1}, respectively. The acidified fraction of the CODin was above 90%, but sludge washout was not significant. These results indicate that the EGSB potentials can be further explored for the anaerobic treatment of low-strength brewery wastewater, even at lower temperatures (Kato et al. 1999).

A significant improvement in UASB system was achieved by modifying the reactor and by operating these modules in series. Akunna and Clark (2000) proposed a hybrid reactor, which was a combination of UASB and an anaerobic baffled reactor for treatment of high-strength wastewaters, referred to as granular bed anaerobic baffled reactor (GRABBR). GRABBR, which combined advantages of a baffled reactor, and a UASB treated whisky distillery waste. Up to 80% of chemical oxygen demand (COD) and 90% of biological oxygen demand (BOD) removal were observed for organic loading rate of 4.75 kg COD $m^{-3}d^{-1}$. Biogas production increased with increasing loading rates from 10 to 22 Ld^{-1} for loading rates 0.99 and 4.75 kg COD $m^{-3}d^{-1}$. The methane content was 60–70%. The effectiveness of the reactor stemmed from the process stability created by phase separation provided by reactor configuration. The system also showed very high solid retention with effluent suspended solid concentration of about 80 mg/L for all organic and hydraulic conditions. This was attributed to the occurrence of granular methanogens in zones downstream of zone occupied by non-granular acidogens.

Upon realising the potential advantages of biphasic biomethanation, Uzal et al. (2003) investigated the anaerobic treatment of whisky distillery waste in two-stage UASB reactors and concluded that the system worked efficiently even at OLRs as high as 39 kg COD $m^{-3}d^{-1}$ resulting in 95–96% COD reduction.

Anaerobic Fluidised Bed Reactors

In the anaerobic fluidised bed reactor, the media for bacterial attachment and growth are kept in the fluidised state by drag forces exerted by the upflowing wastewater. The media used are small particle size sand, activated carbon, etc. Under fluidised state, each medium provides a large surface area for biofilm formation and growth. It enables the attainment of high reactor biomass hold-up and promotes system efficiency and stability. This provides an opportunity for higher organic loading rates and greater resistance to inhibitors. Fluidised bed technology is an effective anaerobic technology for treatment of high-strength wastewater as it favours the transport of microbial cells from the bulk to the surface and thus enhances the contact between the microorganisms and the substrate (Perez et al. 1998). Kida et al. (1995) studied the biological treatment of Shochu distillery wastewater using an anaerobic fluidised bed reactor. By addition of nickel and cobalt, diluting the waste, maximum TOC loading rate of 22 kg TOC $m^{-3}d^{-1}$ could be achieved resulting in 70% TOC reduction. Ability of anaerobic fluidised bed reactor to treat high-strength wastewaters like distillery waste under thermophilic temperatures was studied by Perez et al. (1997). It was confirmed that AFB systems can achieve >82.5% COD reduction at a COD loading of 32.3 kg COD $m^{-3}d^{-1}$ corresponding to hydraulic retention time (HRT) of 0.46 day. The greatest efficiency of substrate removal was 97% for an organic loading rate of 5.9 kg COD $m^{-3}d^{-1}$ and HRT of 2.5 days. The food-to-microorganism (F:M) ratio can be used as a parameter for treatment performance evaluation of AFB. For vinasses, excellent COD reduction and methane production were achievable at the F:M ratio of 0.55 kg COD kg^{-1} VSatt d^{-1} (more than 80% of feed COD was removed, and 9 $m^3m^{-3}d^{-1}$ of methane was produced). Perez-Garcia et al. (2005) compared the performance of two high-rate technologies – upflow anaerobic fixed film reactor and anaerobic fluidised bed reactor – and concluded that the fluidised bed reactor, operated on open-pore sintered glass media, gives total COD removal of 96% at OLR_0 of 5.88 kg COD $m^{-3}d^{-1}$. The anaerobic fluidised bed technology is more effective than the upflow anaerobic fixed film technology, fundamentally as this technology favours the transport of microbial cells from the bulk to the surface and thus enhancing the contact between the microorganism-substrate phases. Application of fluidised bed reactor in principle overcomes mass transfer limitations, but these systems are difficult to manage because of problems of biofilm stability, due to shear stresses or to bed segregation from the inert support material. Moreover, in order to obtain complete fluidisation, the energy requirements of fluidised bed reactors are relatively very high (van Lier et al. 2001).

Anaerobic Batch Reactors

Treatment of distillery waste using batch reactors has not been widely attempted, and potentials, operational feasibility and scaleup of such reactors need to be explored. Treatment of winery wastewater was investigated using an anaerobic sequencing batch reactor (ASBR). The reactor was operated at an organic loading rate (ORL) around 8.6 kg COD $m^{-3}d^{-1}$ with soluble chemical oxygen demand (COD) removal efficiency greater than 98%, hydraulic retention time (HRT) of 2.2d (Ruiz et al. 2002). Banerjee and Biswas (2004) designed a semi-continuous batch digester to investigate biomethanation of distillery waste in mesophilic and thermophilic range of temperatures. The study revealed that there is enormous effect of digestion temperature and substrate concentration in terms of BOD and COD loading on yield of biogas as well as methane content of biogas. Maximum BOD reduction (86.01%), total gas production and methane production (73.23%) occurred at a BOD loading rate of 2.74 kg m^{-3} at 50 °C digestion temperature.

Novel Anaerobic Reactors

Innovative research into bioreactor designs for treatment of high-strength waste like distillery effluent has led to development of novel bioreactors.

To overcome the difficulties of substrate feeding during start-up and to prevent excessive accumulation of volatile fatty acids, Uyanik (2003) developed the split fed anaerobic baffled reactor (SFABR). The potential advantage of the SFABR over the normally fed ABR includes reduction in the severity of conditions (toxicity) in the initial compartments. Split feeding prompted balanced gas production between compartments and improved mixing pattern in the reactor. Distillery effluent was fed into the reactor at an OLR of 10.5 kg COD $m^{-3}d^{-1}$ and after 70 days operation resulted in 90% COD reduction. Arnaiz et al. (2005) designed an invert turbulent bed reactor for treatment of wine distillery waste using pre-colonised bioparticles. The reactor was a modification of inverse fluidised bed showing advantages in terms of better sludge recovery, better liquid recycling, reducing clogging problems and lower energy requirements due to low fluidisation rates. The maximum OLR achieved by the reactor was 28.2 kg COD $m^{-3}d^{-1}$ corresponding to an HRT of 11.2 h and resulting in about 92% COD reduction. Kumar et al. (2007) carried out the biomethanation of distillery spent wash in an anaerobic hybrid reactor (combining sludge blanket and filter) in a continuous mode. The study demonstrated that at optimum HRT of 5 days and at OLR of 8.7 kg COD $m^{-3}d^{-1}$, the COD removal efficiency of the reactor was 79% and concluded that anaerobic hybrid reactor could be successfully employed for treatment of distillery spent wash.

Research into advancing anaerobic treatment technology has been going on for many years. The research has produced many patented systems that provide a variety of advantages in terms of system efficiency, size, capital cost, treatment flexibility, process stability and operating costs. The research into anaerobic digestion continues with efforts to bring into practice outstanding technologies for ecological restoration.

Aerobic Treatment

Anaerobically treated distillery spent wash still contains high concentrations of organic pollutants and as such cannot be discharged directly. Partially treated spent wash has high biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids and high C:N ratio (>20). It can reduce the availability of important mineral nutrients by trapping them into immobile organic forms and may produce phytotoxic substances during decomposition. It is thus unsuitable for irrigation. Treatment comprising physico-chemical methods, adsorption and advanced chemical oxidation processes basically adopted for colour removal in addition to trace organics involves high operational cost. Stringent regulations on discharge of coloured effluent impede direct discharge of anaerobically treated effluent (Nandy et al. 2002). Colourants encountered in sugarcane processing are normally biopolymeric colloidal materials that are negatively charged. All colourants, except caramel, contain phenolics groups in their structures, and phenolics groups contribute to the formation of colourants. IR spectra of alkaline degradative products indicate the presence of ionisable, high molecular weight amino acids. It has been suggested that most of the phenolic colourants are derived from benzoic and cinnamic acid that are precursors of flavonoids, the yellow plant pigments responsible for colour formation. The phenolic acids which form coloured complexes with iron or get oxidised to polymeric colourants are o-hydroxy or o-dihydroxy acids (Mane et al. 2006). During heat treatment, the Maillard reaction takes place resulting in formation of melanoidins, one of the final products of Maillard reaction.

Aerobic treatment of anaerobically treated distillery spent wash has been attempted for the decolourisation of the major colourant, melanoidins, and for further reduction of the organics and thus reducing the chemical oxygen demand (COD) and biological oxygen demand (BOD) further. A large number of microorganisms such as bacteria (pure and mixed culture), cyanobacteria, yeast and fungi have been isolated in recent years that are capable to degrade melanoidins and thus decolourise the waste.

Fungal Treatment

Fungi are recognised by their superior aptitude to produce a large variety of extracellular proteins,

organic acids and other metabolites and for capacity to adapt to severe environmental constraints (Coulibaly et al. 2003). Increasing attention has been directed towards utilising microbial activity for decolourisation of molasses spent wash, and several reports have indicated that some fungi in particular have potential for use in this purpose (Kumar et al. 1998). Some of the most studied fungi having the ability to degrade and decolourise distillery effluent are *Aspergillus fumigatus* G-2–6, *A. niger, A. niveus* and *A. fumigatus* $U_{B2}60$ which brought about an average of 69–75% decolourisation along with 70–90% COD reduction (Ohmomo et al. 1987; Miranda et al. 1996; Jimnez et al. 2003; Shayegan et al. 2005; Angayarkanni et al. 2003; Mohammad et al. 2006). Treatment of distillery spent wash with ascomycetes group of fungi such as *Penicillium* spp., *Penicillium decumbens* and *Penicillium lignorum* resulted in about 50% colour and COD reduction and 70% phenol removal (Jimnez et al. 2003). Sirianuntapiboon et al. (1995) reported an absorption mechanism for decolourisation of melanoidins by *Rhizoctonia* spp. D-90. The fungal culture decolourised the molasses medium as well as the synthetic melanoidin medium by absorption of melanoidin pigment by the cells as a macromolecule and its intracellular accumulation in the cytoplasm and around the cell membrane as melanoidin complex, which was then gradually decolourised by intracellular enzymes.

White-rot fungi is another group of one of the most widely exploited microorganism in distillery effluent bioremediation. White-rot fungi produce various isoforms of extracellular oxidases including laccases, manganese peroxidases and lignin peroxidase, which are involved in the degradation of lignin in their natural lignocellulosic substrate. This ligninolytic system of white-rot fungi is directly involved in the degradation of various xenobiotic compounds and dyes (Wesenberg et al. 2003). Table 14.2 gives details about different white-rot fungi employed in decolourisation of distillery effluent and the role of different enzymes in the process.

Recently, Pant and Adholeya (2007b) isolated three fungal cultures and identified them by using molecular approaches as *Penicillium pinophilum* TERI DB1, *Alternaria gaisen* TERI DB6, and *Pleurotus florida* EM 1303. These cultures were found to produce ligninolytic enzymes and decolourised the effluent up to 50, 47, and 86%, respectively.

Bacterial Treatment

Different bacterial cultures have been isolated capable of both bioremediation and decolourisation of anaerobically treated distillery spent wash. Kumar and Viswanathan (1991) isolated bacterial strains from sewage, and these strains were able to reduce the COD of the distillery effluent by 80% after 4–5 days. Carbon dioxide, volatile acids and biomass were the products of biodegradation of the effluent. Kumar et al. (1997) isolated a facultative anaerobic pure bacterial culture L-2, a Gram-positive nonmotile rod belonging to genus Lactobacilli, which was able to decolourise the effluent by 31% and remove 57% COD of 12.5% diluted wastewater supplemented with $10gL^{-1}$ glucose in 7 days. Nakajima-Kambe et al. (1999) isolated a *Bacillus* sp. which decolourised molasses wastewater up to 35.5% within 20 d at 55 °C (thermophilic conditions) under anaerobic conditions. The molecular weight distribution as determination by gel permeation chromatography revealed that there was decrease in colour contributing small molecules as well as large molecules. Some researchers carried out melanoidin decolourisation by using immobilised whole cells. Ohmomo et al. (1988) used calcium alginate-immobilised cells of *Lactobacillus hilgardii* to decolourise melanoidin solution which resulted in 40% decolourisation. Decolourisation of molasses wastewater by immobilised cells of *Pseudomonas fluorescens* on porous cellulose carrier was attempted achieving 76% decolourisation in 24 h at 30°C. Cellulose carrier coated with collagen was found to be most efficient carrier, which could be reused with 50% decolourisation activity retained until the seventh day (Dahiya et al. 2001a). Jain et al. (2001) isolated three bacterial cultures from the activated sludge of a distillery wastewater plant identified as *Xanthomonas*

Table 14.2 White-rot fungi employed in treatment of distillery effluent treatment

Culture	Treatment	COD removal	Colour removal	Enzymes	Reference
Phanerochaete chrysosporium	Free cells as well as Ca alginate-immobilised cells decolourised the distillery effluent	NR	85% (free) 59% (immobilised)	NR	Fahy et al. (1997)
Coriolus sp. No. 20	Synthetic melanoidin solution was decolourised by the fungus	NR	80%	Sorbose oxidase	Watanabe et al. (1982)
P. chrysosporium	Synthetic melanoidins as well as natural melanoidins were decolourised in presence of glucose and peptone	NR	80%	Extracellular enzyme required presence of glucose for decolourisation	Dahiya et al. (2001b)
Trametes versicolor	Anaerobically treated distillery effluent supplemented with sucrose and inorganic N sources was decolourised by the culture in shake flask studies	75%	80%	NR	Benito et al. (1997)
P. chrysosporium	Both the cultures decolourised to reduced the COD of effluent in presence of (3–5%) glucose and 0.1% yeast extract	73%	53.5%	NR	Kumar et al. (1998)
Coriolus versicolor		70%	71.5%	NR	
Flavodon flavus	Distillery effluent was decolourised using these marine basidiomycetes in presence of 5% glucose	NR	80%	Glucose oxidase accompanied with hydrogen peroxide	Raghukumar and Rivonkar (2001), Raghukumar et al. (2004).
Coriolus hirsutus IF044917	The fungal culture was immobilised on PUF and used for decolourisation of melanoidins present in heat-treated liquor	NR	45%	NR	Fujita et al. (2000)

C. hirsutus	Synthetic as well as wastewater melanoidin was decolourised by the fungus in a medium containing glucose and peptone	NR	80%	MiP and MnP and presence of extracellular H_2O_2	Miyata et al. (1998), (2000)
Coriolus versicolor	The cultures were incubated along with cotton stalks in vinasses media in static condition. No synthetic carbon or nitrogen sources were used	49	63	NR	Kahraman and Yesilada (2003)
Funalia trogii		62	57		
P. chrysosporium		57	37		
P. pulmonarius		34	43		
P. chrysosporium 1557	Decolourisation of MWW was studied. Effect of veratryl alcohol and Mn (II) on decolourisation was also studied	NR	75%	LiP and MnP	Vahabzadeh et al. (2004)
P. chrysosporium NCIM 1073	The cultures were employed to study the decolourisation of molasses in medium containing 2% w/w glucose in static as well as submerged conditions	NR	Nil	Nil	Thakkar et al. (2006)
NCIM 1106		NR	82%	LiP and MnP	
NCIM 1197		NR	76%	LiP and MnP	
P. chrysosporium ATCC 24725	The fungus was immobilised on different support materials such as PUF and scouring wet, and the decolourisation was carried out in a RBC	48%	55%	NR	Guimaraes et al. (2005)

fragariae, *B. megaterium* and *B. cereus* which were found to remove COD and colour from the distillery effluent in the range of 55–68% and 38–58%, respectively. Two bacterial strains *Pseudomonas putida* U and *Aeromonas* strain Ema, in a two-stage bioreactor, were used to bioremediate anaerobically treated distillery spent wash. In the first stage, *P. putida* reduced the COD and colour by 44.4 and 60%. The *Aeromonas strain Ema*, in the second stage, reduced the COD by 44%. Algal bioassay was used to evaluate the quality of the spent wash before and after treatment. The spent wash was eutrophic before experimental treatment, but, after treatment, it showed poor algal growth (Ghosh et al. 2002). In another study, Ghosh et al. (2004) isolated various bacterial strains capable of using recalcitrant compounds of molasses spent wash as sole carbon source and thus reducing the COD of the waste. The microorganisms were identified by molecular methods like 16S rRNA sequencing, and their phylogenetic relationship elucidated. Six strains, namely, *Pseudomonas*, *Enterobacter*, *Aeromonas*, *Stenotrophomonas*, *Acinetobacter* and *Klebsiella* brought about 44% COD reduction of the distillery effluent; however, no decolourisation was observed. Sirianuntapiboon et al. (2004) isolated an acetogenic strain from vegetable and juice samples which decolourised the molasses pigment medium and anaerobically treated distillery effluent to 73–76% within 5 days when supplemented with glucose and nitrogen sources. In replacement culture system involving six replacements, the strain showed constant decolourisation and decrease in BOD and COD values of 58.5–82.2% and 35.5–71.2%, respectively.

Sangave and Pandit (2006a) proposed a combined treatment technique consisting of enzymatic hydrolysis by cellulases followed by aerobic oxidation with a Gram-positive culture ASN 6. The rate of aerobic oxidation was enhanced by 2.3-fold for pretreated sample as compared to untreated sample. In another study, Sangave and Pandit (2006b) used a combination of irradiation with ultrasound and hydrolysis with cellulase prior to aerobic oxidation with ASN 6 which resulted in fourfold increase in the initial oxidation rate over the untreated batch of effluent.

Mixed culture studies have been carried out by several researchers for degradation of different effluents such as textile effluents. As the catabolic activities of microorganisms in a mixed consortium complement each other, obviously syntrophic interactions present in mixed communities lead to complete mineralisation of the effluent (Moosvi et al. 2007). The decolourisation of synthetic melanoidins (i.e. GGA, GAA, SGA and SAA) by three *Bacillus* isolates, *Bacillus thuringiensis* (MTCC 4714), *Bacillus brevis* (MTCC 4716) and *Bacillus* sp. (MTCC 6506), was studied by Kumar and Chandra (2006). Significant reduction in the values of physico-chemical parameters was noticed along with the decolourisation of all four melanoidins (10% v/v). *B. thuringiensis* (MTCC 4714) caused maximum decolourisation followed by *B. brevis* (MTCC 4716) and *Bacillus* sp. (MTCC 6506). A mixed culture comprised of these three strains was capable of decolourising all four melanoidins. The medium that contained glucose as a sole carbon source showed 15% more decolourisation than that containing both carbon and nitrogen sources. Melanoidin SGA was maximally decolourised (50%), while melanoidin GAA was decolourised least (6%) in the presence of glucose as a sole energy source. The addition of 1% glucose as a supplementary carbon source was essential for co-metabolism of melanoidin complex. The decolourisation of synthetic melanoidin by three *Bacillus* sp. significantly reduced the toxicity to the tubificid worm (*Tubifex tubifex*, Müller). Chaturvedi et al. (2006) isolated and characterised 15 culturable rhizosphere bacteria of *Phragmites australis* growing in distillery effluent-contaminated sites. These 15 cultures were *Microbacterium hydrocarbonoxydans*, *Achromobacter xylosoxidans*, *Bacillus subtilis*, *B. megaterium*, *B. anthracis*, *B. licheniformis*, *A. xylosoxidans*, *Achromobacter* sp., *B. thuringiensis*, *B. licheniformis*, *B. subtilis Staphylococcus epidermidis*, *Pseudomonas migulae*, *Alcaligenes faecalis* and *B. cereus* which collectively brought about 76% decolourisation and 85–86% BOD and COD reduction of the effluent within 30 days. A bacterial consortium comprising of *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophilia* and *Proteus mirabilis* isolated from distillery effluent-contaminated sites exhibited 67%

decolourisation and 51% COD reduction within 72 h in presence of glucose and basal salts (Mohana et al. 2007).

Cyanobacterial/Algal Treatment

Cyanobacteria are considered ideal for treatment of distillery effluent as they apart from degrading the polymers also oxygenate water bodies, thus reducing the BOD and COD. Kalavathi et al. (2001) explored the possibility of using a marine cyanobacterium for decolourisation of distillery spent wash and its ability to use melanoidins as carbon and nitrogen source. A marine filamentous, non-heterocystous form *Oscillatoria boryana* BDU 92181 used the recalcitrant biopolymer melanoidin as nitrogen and carbon source leading to decolourisation. Indirect evidence through the study of nitrogen assimilating enzymes as well as direct evidence of using ^{14}C radiolabeled synthetic melanoidins confirms this ability. The organism decolourised pure melanoidin pigment (0.1% W/V) by about 75% and crude pigment in the distillery effluent (5% V/V) by about 60% in 30 days. The mechanism of colour removal is postulated to be due to the production of hydrogen peroxide, hydroxyl anions and molecular oxygen, released by the cyanobacterium during photosynthesis. Valderrama et al. (2002) studied the feasibility of combining microalgae *Chlorella vulgaris* and macrophyte *Lemna minuscula* for bioremediation of wastewater from ethanol-producing units. This combination resulted in 61% COD reduction and 52% colour reduction. First, the microalgal treatment led to removal of organic matter, and further treatment with macrophytes removed other organic matter and colour and precipitated the microalgae.

Conclusion

Distillery effluent has been treated by implementing different bioremediation technologies like anaerobic digestion using various reactor designs and aerobic treatment with bacteria, fungi and other microorganisms. However, these bioremediation technologies have their pros and cons which have been mentioned in the chapter. A common feature of these methods is their relative cost and for some the simultaneous creation of other hazardous by-products. Hence, there exists a need to develop a comprehensive treatment technology which is not only cost-effective and ecofriendly but also meets the discharge quality standards of pollution control boards.

References

Acharya BK, Mohana S, Madamwar D (2008) Anaerobic treatment of distillery spent wash – a study on upflow anaerobic fixed film bioreactor. Bioresour Technol 99:4621–4626

Akunna JC, Clark M (2000) Performance of a granular-bed anaerobic baffled reactor (GRABBR) treating whisky distillery wastewater. Bioresour Technol 74:257–261

Angayarkanni J, Palaniswamy M, Swaminathan K (2003) Biotreatment of distillery effluent using *Aspergillus niveus*. Bull Environ Contam Toxicol 70:268–277

Arnaiz C, Elmaleh S, Lebrato J, Moletta R (2005) Start up of an anaerobic inverse turbulent bed reactor fed with wine distillery wastewater using pre-colonized bioparticles. Water Sci Technol 51:153–158

Banerjee S, Biswas GK (2004) Studies on biomethanation of distillery waste and its mathematical analysis. Chem Eng J 102:193–201

Benito GG, Miranda MP, de los Santos DR (1997) Decolourization of wastewater from alcoholic fermentation process with *Trametes versicolor*. Bioresour Technol 61:33–37

Berg C (2004) World fuel ethanol analysis an outlook. www.distill.com

Borja R, Sanchez E, Martin A, Jimenez AM (1996) Kinetic behaviour of waste tyre rubber as microorganism support in an anaerobic digester treating cane molasses distillery slops. Bioprocess Biosyst Eng 16:17–23

Chandraraj K, Gunasekaran P (2004) Bacterial alcoholic fermentation. In: Ashok P (ed) Concise encyclopedia of bioresource technology. Food Products Press, New York, pp 327–333

Chaturvedi S, Chandra R, Rai V (2006) Isolation and characterization of *Phragmites australis* (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. Ecol Eng 27:202–207

Coulibaly L, Germain G, Spiros A (2003) Utilization of fungi for biotreatment of raw wastewaters. Afr J Biotechnol 2:620–630

Dahiya J, Singh D, Nigam P (2001a) Decolorization of molasses wastewater by cells of *Pseudomonas fluorescens immobilized* on porous cellulose carrier. Bioresour Technol 78:111–114

Dahiya J, Singh D, Nigam P (2001b) Decolorization of synthetic and melanoidins using the white rot fungus

Phanerochaete chrysosporium JAG-40. Bioresour Technol 78:95–98

Dhembare AJ, Amin S (2002) Effect of distillery waste on soil properties. Eco Environ Conserv 8:297–299

Fahy V, FitzGibbon FJ, McMullan G, Singh D, Marchant R (1997) Decolorization of molasses spent wash by *Phanerochaete chrysosporium*. Biotechnol Lett 16:97–99

Fang HHP, Chui HK, Li YY (1994) Microbial structure and activity of UASB granules treating different wastewaters. Water Sci Technol 30:87–96

Florencio L, Field JA, Lettinga G (1997) High rate anaerobic treatment of alcoholic wastewaters. Braz J Chem Eng 14(4), [online]. Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-66321997000400016&lng=en&nrm=iso>. ISSN 0104-6632. http://dx.doi.org/10.1590/S0104-66321997000400016

Fujita M, Era A, Ike M, Soda S, Miyata N, Hirao T (2000) Decolorization of heat treatment liquor of waste sludge by a bioreactor using polyurethane foam immobilized white rot fungus equipped with an ultramembrane filtration unit. J Biosci Bioeng 90:387–394

Ghangrekar MM, Joshi SG, Asolekar SR (2003) Upflow anaerobic sludge blanket (UASB) process: low cost biological wastewater treatment. In: Goel PK (ed) Advances in industrial wastewater treatment. ABD Publishers, Jaipur, pp 368–379

Ghosh S (1990) Principles and potentials of biphasic fermentation. In: International conference on biogas: technologies and implementation strategies, Pune, India, 10–15 Jan 1990

Ghosh M, Ganguli A, Tripathi AK (2002) Treatment of anaerobically digested distillery spentwash in a two stage bioreactor using *Pseudomonas putida* and *Aeromonas sp.*. Process Biochem 7:857–862

Ghosh M, Verma SC, Mengoni A, Tripathi AK (2004) Enrichment and identification of bacteria capable of reducing chemical oxygen demand of anaerobically treated molasses spent wash. J Appl Microbiol 96:1278–1286

Goodwin JAS, Stuart JB (1994) Anaerobic digestion of malt whisky distillery pot ale using upflow anaerobic sludge blanket reactor. Bioresour Technol 49:75–81

Guimaraes C, Porto P, Oliveira R, Mota M (2005) Continuous decolourization of a sugar refinery wastewater in a modified rotating biological contactor with *Phanerochaete chrysosporium* immobilized on polyurethane foam discs. Process Biochem 40:535–540

Handa BK, Seth R (1990) Waste management in distillery industry. J IAEM 17:44–54

Harada H, Uemura S, Chen A, Jayadevan J (1996) Anaerobic treatment of a recalcitrant distillery wastewater by a thermophilic UASB reactor. Bioresour Technol 55:215–223

Jain MK, Bhatnagar L, Zeikus JG (1990) Biochemical pathways for methane fermentation and use of granulated biomass for high rate anaerobic digestion. International conference on biogas: technologies and implementation strategies, Pune, India, 10–15 Jan 1990

Jain N, Nanjundaswamy C, Minocha AK, Verma CL (2001) Isolation, screening and identification of bacterial strains for degradation of predigested distillery wastewater. Indian J Exp Biol 39:490–492

Jhung JK, Choi E (1995) A comparative study of UASB and anaerobic fixed film reactors with development of sludge granulation. Water Res 29:271–277

Jimnez AM, Borja R, Martin A (2003) Aerobic-anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. Process Biochem 38:1275–1284

Joshi HC (1999) Bio energy potential of distillery effluents. Bio Energ News 3:10–15

Kahraman S, Yesilada O (2003) Decolorization and bioremediation of molasses wastewater by white rot fungi in a semi solid state condition. Folia Microbiol 48:525–528

Kalavathi DF, Uma L, Subranian G (2001) Degradation and metabolism of the pigment-melanoidin in distillery effluent by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. Enzyme Microb Technol 29:246–251

Kalyuzhnyi SV, Martinez E, Martinez J (1997) Anaerobic treatment of high strength cheese whey wastewater in laboratory and pilot scale UASB reactors. Bioresour Technol 60:59–65

Kalyuzhnyi SV, Gladchenko MA, Sklyar VI, Kizimenko YS, Shcherbakov SS (2001) One- and two-stage upflow anaerobic sludge-bed reactor pretreatment of winery wastewater at 4–10 °C. Appl Biochem Biotechnol 90:107–124

Kato MT, Rebac S, Lettinga G (1999) Anaerobic treatment of low-strength brewery wastewater in expanded granular sludge bed reactor. Appl Biochem Biotechnol 76:15–32

Kida K, Morimura S, Abe N, Sonoda Y (1995) Biological treatment of Shochu distillery wastewater. Process Biochem 30:125–132

Kleerebezem R, Macarie H (2003) Treating industrial wastewater: anaerobic digestion comes of age. www.che.com

Kumar P, Chandra R (2006) Decolorization and detoxification of synthetic molasses melanoidins by individual and mixed cultures of Bacillus spp. Bioresour Technol 97:2096–2102

Kumar S, Viswanathan L (1991) Production of biomass, carbon dioxide, volatile acids and their interrelationship with decrease in chemical oxygen demand, during distillery waste treatment by bacterial strains. Enzyme Microb Technol 13:179–186

Kumar V, Wati L, FitzGibbon F, Nigam P, Banat IM, Singh D, Marchant R (1997) Bioremediation and decolorization of anaerobically digested distillery spent wash. Biotechnol Lett 19:311–313

Kumar V, Wati L, Nigam P, Banat IM, Yadav BS, Singh D, Marchant R (1998) Decolorization and biodegradation of anaerobically digested sugarcane molasses spent wash effluent from biomethanation plants by white rot fungi. Process Biochem 33:83–85

Kumar GS, Gupta SK, Singh G (2007) Biodegradation of distillery spent wash in anaerobic hybrid reactor. Water Res 41:721–730

Lalov IG, Krysteva MA, Phelouzat JL (2001) Improvement of biogas production from vinasse via covalently immobilized methanogens. Bioresour Technol 79:83–85

Lata K, Kansal A, Balakrishnan M, Rajeshwari KV, Kishore VVN (2002) Assessment of biomethanation potential of selected industrial organic effluents in India. Resour Conserv Recy 35:147–161

Lettinga G (1990) Principles and potentials of the UASB- process. International conference on biogas: technologies and implementation strategies, Pune, India, 10–15 Jan 1990

Lettinga G, Hulshoff Pol LW (1991) UASB process design for various types of wastewaters. Water Sci Technol 24:87–107

Mailleret L, Bernard O, Steyer JP (2003) Robust regulation of anaerobic digestion process. Water Sci Technol 48:87–94

Mane JD, Modi S, Nagawade S, Phadnis SP, Bhandari VM (2006) Treatment of spentwash using chemically modified bagasse and colour removal studies. Bioresour Technol 97:1752–1755

Matkar LS, Gangotri MS (2003) Acute toxicity tests of sugar industrial effluents on the freshwater crab, *Barytelphusa guerini* (H. Milne Edwards) (Decapoda, Potamidae). Pollut Res 22:269–276

Michaud S, Bernet N, Buffiere P, Roustan M, Moletta R (2002) Methane yield as a monitoring parameter for the start up of anaerobic fixed film reactors. Water Res 36:1385–1391

Miranda PM, Benito GG, Cristobal NS, Nieto CH (1996) Color elimination from molasses wastewater by *Aspergillus niger*. Bioresour Technol 57:229–235

Miyata N, Iwahori K, Fujita M (1998) Manganese independent and dependent decolorization of melanoidin by extracellular hydrogen peroxide and peroxidases from *Coriolus hirsutus* pellets. J Ferment Bioeng 85:550–553

Miyata N, Mori T, Iwahori K, Fujita M (2000) Microbial decolorization of melanoidin containing wastewaters: combined use of activated sludge and the fungus *Coriolus hirsutus*. J Biosci Bioeng 89:145–150

Mohammad P, Azarmidokht H, Fatollah M, Mahboubeh B (2006) Application of response surface methodology for optimization of important parameters in decolorizing treated distillery wastewater using Aspergillus fumigatus UB2 60. Int Biodeter Biodegr 57:195–199

Mohana S, Desai D, Madamwar D (2007) Biodegradation and decolorization of anaerobically treated distillery spent wash by a novel bacterial consortium. Bioresour Technol 98:333–339

Moosvi S, Kher X, Madamwar D (2007) Isolation, characterization and decolorization of textile dyes by a mixed bacterial consortium JW-2. Dyes Pigments 74:723–729

Nagamani B, Ramasamy K (1999) Biogas production technology: an Indian perspective. Curr Sci 77:44–55

Nakajima KT, Shimomura M, Nomura N, Chanpornpong T, Nakahara T (1999) Decolorization of molasses wastewater by Bacillus sp. under thermophilic and anaerobic conditions. J Biosci Bioeng 87:119–121

Nandy T, Shastry S, Kaul SN (2002) Wastewater management in a cane molasses distillery involving bioresource recovery. J Environ Manage 65:25–38

Ogbonna JC (2004) Fuel ethanol production from renewable biomass resources. In: Pandey A (ed) Concise encyclopedia of bioresource technology. Food Products Press, New York, pp 346–361

Ohmomo S, Kaneko Y, Sirianuntapiboon S, Somchal P, Atthasampunna P, Nakamura I (1987) Decolorization of molasses wastewater by a thermophilic strain, *Aspergillus fumigatus* G-2-6. Agric Biol Chem 51:3339–3346

Ohmomo S, Daengsabha W, Yoshikawa H, Yui M, Nozaki K, Nakajima T, Nakamura I (1988) Screening of anaerobic bacteria with the ability to decolourize molasses melanoidin. Agric Biol Chem 57:2429–2435

Pandey RA, Malhotra A, Tankhiwale S, Pande S, Pathe PP, Kaul SN (2003) Treatment of biologically treated distillery effluent – a case study. Int J Environ Stud 60:263–275

Pant D, Adholeya A (2007a) Biological approaches for treatment of distillery wastewater: a review. Bioresour Technol 98:2321–2334

Pant D, Adholeya A (2007b) Identification, ligninolytic enzyme activity and decolorization potential of two fungi isolated from a distillery effluent contaminated site. Water Air Soil Pollut 183:165–176

Patel H, Madamwar D (2000) Biomethanation of low pH petrochemical wastewater using up-flow fixed film anaerobic bioreactors. World J Microbiol Biotechnol 16:69–75

Patel H, Madamwar D (2002) Effects of temperatures and organic loading rates on biomethanation of acidic petrochemical wastewater using an anaerobic upflow fixed-film reactor. Bioresour Technol 82:65–71

Pathade GR (2003) A review of current technologies for distillery wastewater treatment. In: Goel PK (ed) Advances in industrial wastewater treatment. ABD Publishers, Jaipur, pp 180–239

Pathak H, Joshi HC, Chaudhary A, Kalra N, Dwivedi MK (1999) Soil amendment with distillery effluent for wheat and rice cultivation. Water Air Soil Pollut 113:133–140

Perez M, Romero LI, Nebot E, Sales D (1997) Colonisation of a porous sintered glass support in anaerobic thermophilic bioreactors. Bioresour Technol 59:177–183

Perez M, Romero LI, And Sales D (1998) Comparative performance of high rate anaerobic thermophilic technologies treating industrial wastewater. Water Res 32:559–564

Perez-Garcia M, Romero-Garcia LI, Rodriguez-Cano R, Sales-Marquez D (2005) Effect of the pH influent conditions in fixed film rectors for anaerobic thermophilic treatment of wine-distillery wastewater. Water Sci Technol 51:183–189

Raghukumar C, Rivonkar G (2001) Decolourization of molasses spent wash by white rot fungus Flavodon flavus, isolated from a marine habitat. Appl Microbiol Biotechnol 55:510–514

Raghukumar C, Mohandass C, Kama S, Shailaja MS (2004) Simultaneous detoxification and decolourisation of molasses spent wash by the immobilized white-rot fungus *Flavodon flavus* isolated from a marine habitat. Enzyme Microb Technol 35:197–202

Rajeshwari KV, Balakrishnan M, Kansal A, Lata K, Kishore VVN (2000) State-of art of anaerobic digestion technology for industrial wastewater treatment. Renew Sustain Energy Rev 4:135–156

Ramakritinan CM, Kumaraguru AK, Balasubramanian MP (2005) Impact of distillery effluent on carbohydrate metabolism of freshwater fish, *Cyprinus carpio*. Ecotoxicology 14:693–707

Rao SB (1972) A low cost waste treatment method for disposal of distillery waste (spent wash). Water Res 6:1275–1282

Ruiz C, Torrijos M, Sousbie P, Lebrato Martinez J, Moletta R, Delgenes JP (2002) Treatment of winery wastewater by an anaerobic sequencing batch reactor. Water Sci Technol 45:219–224

Saha NK, Balakrishnan M, Batra VS (2005) Improving industrial water use: case study for an Indian distillery. Resour Conserv Recy 43:163–174

Sangave PC, Pandit AB (2006a) Enhancement in biodegradability of distillery wastewater using enzymatic pretreatment. J Environ Manag 78:77–85

Sangave PC, Pandit AB (2006b) Ultrasound and enzyme assisted biodegradation of distillery wastewater. J Environ Manag 80:36–46

Satyawali Y, Balakrishnan M (2007) Removal of color from biomethanated distillery spent wash by treatment with activated carbons. Bioresour Technol 98:2629–2635

Saxena KK, Chauhan RRS (2003) Oxygen consumption in fish, *Labeo rohita* (HAM.) caused by distillery effluent. Ecol Environ Conserv 9:357–360

Seth R, Goyal SK, Handa BK (1995) Fixed film biomethanation of distillery spentwash using low cost porous media. Resour Conserv Recy 14:79–89

Sharma J, Singh R (2000) Characterization of sludge from UASB reactors operating on molasses based distillery effluent. Indian J Microbiol 40:203–205

Shayegan J, Pazouki M, Afshari A (2005) Continuous decolorization of anaerobically digested distillery wastewater. Process Biochem 40:1323–1329

Singh PN, Robinson T, Singh D (2004) Treatment of industrial effluents – distillery effluent. In: Pandey A (ed) Concise encyclopedia of bioresource technology. Food Products Press, New York, pp 135–141

Sirianuntapiboon S, Sihanonth P, Somchai P, Atthasampunna P, Hayashida S (1995) An adsorption mechanism for melanoidin decolorization by *Rhizoctonia* sp. Biosci Biotechnol Biochem 59:1185–1189

Sirianuntapiboon S, Phothilangka P, Ohmomo S (2004) Decolorization of molasses wastewater by a strain no. BP103 of acetogenic bacteria. Bioresour Technol 92:31–39

Thakkar AP, Dhamankar VS, Kapadnis BP (2006) Biocatalytic decolourisation of molasses by *Phanerochaete chrysosporium*. Bioresour Technol 97:1377–1381

Uyanik S (2003) A novel anaerobic reactor: split fed anaerobic baffled reactor (SFABR). Turkish J Eng Environ Sci 27:339–345

Uzal N, Gokcay CF, And Demirer GN (2003) Sequential (anaerobic/aerobic) biological treatment of malt whisky wastewater. Process Biochem 39:279–286

Vahabzadeh F, Mehranian M, Saatari AR (2004) Color removal ability of *Phanerochaete chrysosporium* in relation to lignin peroxidase and manganese peroxidase produced in molasses wastewater. World J Microbiol Biotechnol 20:859–864

Valderrama LT, Del Campo CM, Rodriguez CM, Bashan LE, Bashan Y (2002) Treatment of recalcitrant wastewater from ethanol and citric acid using the microalga *Chlorella vulgaris* and the macrophyte *Lemna minuscula*. Water Res 36:4185–4192

Van Lier JB, Tilche A, Ahring BK, Macarie H, Moletta R, Dohanyos M, Hulshoff Pol LW, Lens P, Verstraete W (2001) New perspectives in anaerobic digestion. Water Sci Technol 43:1–18

Watanabe Y, Sugi R, Tanaka Y, Hayashida S (1982) Enzymatic decolorization of melanoidin by *Coriolus sp.*. Agric Biol Chem 46:1623–1630

Wesenberg D, Kyriakides I, Agathos SN (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187

Wolmarans B, de Villiers GH (2002) Start up of an effluent treatment plant on distillery wastewater. Water SA 28:63–68

Application of Natural Dyes: An Emerging Environment-Friendly Solution to Handmade Paper Industry

15

Saakshy, A.K. Sharma, and R.K. Jain

Abstract

The handmade paper industry is one of the most prominent export-oriented sectors of India. Handmade paper means "totally eco-friendly paper" crowned with a trademark of eco-friendliness, utilizing nonwoody raw material for handmade papermaking. In the present scenario, there is global consciousness about ecology and environment due to alarming "ozone hole" and "greenhouse effect" due to deforestation. It is said that 27 trees are being cut to make 1 Tonne of paper based on 45% yield, 50% moisture, and 6″ girth × 30″ ht. The main requirement is use of paper which is made by using nonwoody raw material along with eco-friendly pulping process, i.e., it should be acid-free paper, totally chlorine-free paper, and azo-free paper. The conventional direct dyes are being used for making colored handmade paper. Some of the direct dyes have possibility of having azo group (–N=N–) which on reduction releases carcinogenic or harmful amines. Azo dyes are metabolized to the corresponding amines by liver enzymes. Aromatic amines are easily absorbed by the skin. Twenty-two harmful amines have been identified so far. The use of benzidine-based dyes has been stopped in most of the countries in order to avoid workers from coming in its contact as the benzidine-based dyes have been reported to be carcinogenic. The Indian handmade industry is now facing the impact of various stringent parameters set forth by developing countries for the protection of environment. One of the main requirements is that the dye used for coloring the paper should be azo-free.

Keywords

Handmade paper • Eco-friendly • Dyes • Azo dyes • Natural dyes

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Introduction

The history of paper dates back to the history of human culture and civilization. The Egyptians, Greeks, and Romans wrote on "papyrus," a paperlike material. Today's kind of paper was first developed and used in China. Since its birth the paper was the most important carrier of information in the past. Growth of population and need to transfer the knowledge, education, and information to the society at large were the forcing factors for stupendous increase in the production of paper. In former times, paper was a valuable product and papermaking an art – that was often kept secret because of the outstanding advantages of the product.

The history of handmade paper in India dates back to the third century BC. Making handmade paper is a traditional art that has been practiced by a particular class of people for generations together. This art has been passed on from one generation of craftsmen to another. These craftsmen are known as "Kagzis." Their name is derived from the Urdu word "kavas," which means paper.

Under the British, handmade paper received a serious setback as the British encouraged the import of mill-made paper from the Western countries. At this point, Mahatma Gandhi provided them with the much-needed support by buying handmade paper in bulk for his Ashram and other associates. After independence, the Khadi and Village Industries Commission (KVIC) included handmade paper in the list of crafts to be promoted. Over the years, the handmade paper industry has grown slowly but steadily and is today a major player in the world market, exporting a major portion of its production.

It will not be exaggeration to say that today handmade paper has emerged as one of the prominent sectors in export market. The sector is spreading its feathers, and this can be visibly seen by increasing demand and production of handmade paper in domestic as well as in export market. As the world is becoming more environment conscious, the obsession for handmade paper is increasing day by day. So its use creates a sense of our credibility to our environment along with pleasing our visual sense.

For 1mt of paper, trees of eucalyptus required 27 (based on 45% yield, 50% moisture, 6″ girth 30′ ht.).

Coupled with increased literacy and modernization, the per capita consumption of paper has increased from 4.5 kg in late 1990s to around 7.0 kg in the recent years. Handmade paper utilizes nonwoody raw material for making paper, so it plays significant role in maintaining the ecology.

Alarming environment problems have shaken the world to think about our environment before it is too late. Fortunately, developed countries have taken a step forward and set norms at manufacturing/product making/user's end for their environment friendliness. Handmade paper, not an exemption, has also to prove its eco-friendliness in terms of certain criteria satisfying its eco-friendliness as given below:

- Use of nonwoody raw material
- Eco-friendly pulping process
- Eco-friendliness of paper after addition of chemical additives
- Nonpolluting process

The eco-friendliness of handmade paper includes nature of dyes, acidity/alkalinity of paper, and presence of azo group in dye.

In the present milieu, concern for the environment has created an escalating interest in natural dyes. It is common belief that natural dyes are friendlier to the environment than synthetic dyes. Moreover, when the question of fastness is raised, it is to note that all museum textiles predating 1856 are naturally dyed. Even after so many years, their colors remain rich, vibrant, beautiful, and inspiring. Dyes which were in use in Greco-Roman times and in the middle ages were almost entirely natural dyes. In India, the most important mineral pigments analyzed from paintings on walls, cloth, and paper have been natural dyes. The depictions on wall paintings of Ajanta, Ellora, and other sites indicate that early Indian very well knew dyeing and painting on fabric. The dyers, printers, and Ayurvedic practitioners with their vast experience and skills have contributed much to natural dyes. They tried each and every part of plants like roots, heartwood, branches, leaves, flowers, pods, buds, and fruits. In spite of tremendous efforts, it has been

felt that little scientific advancement has been made in vegetable dyes. The reason might be advent of synthetic dyes after 1856 dominated the world trade of colors and substituted vegetable dyes to a very good extent. The use of vegetable dyes continued only in few pockets of India as a small industry (Ethel Mairet and Pepler 1916).

Dyes

Dye is very important aspect in handmade papermaking. A simple method of applying dyes to cotton fibers involves the dissolution of dye in water and then adding the filtered solution of dye to fiber suspension in beater. Since dyeing is done directly on cotton, dyes belonging to this class are called direct dye. In handmade papermaking the conventional dye used is direct dye due to its more fastness to light and strong affinity for cellulose.

What Is Fastness to Light?

When a dyed paper is exposed to sunlight during its drying or use, the dye should not fade or change its color that shows good fastness to light. During exposure to sunlight the dye absorbs some portion of sunlight, the absorbed light has certain amount of energy, and the absorbed energy may bring out self-destruction of the dye in the presence of oxygen and moisture. Instrument named as Xenon Arc tester (Company, country) is used to evaluate the fastness to light.

What Are Azoic Dyes?

Direct dyes having azo group (–N=N-) are called azoic dyes. Azo dyes release harmful amines which are allergic, carcinogenic, or poisonous in nature. Germany is very specific about the use of Azo dyes which on reduction release carcinogenic or harmful amines. Twenty-two harmful amines are found so far by Germany. Germany was the first country to impose a comprehensive ban on presence of azo dyes containing harmful amines or which are reduced to carcinogenic aryl amines. Many other countries followed this ban. The benzidine-based dyes were stopped in almost all countries to stop workers from coming in contact with benzidine which causes cancer. The presence of banned amines is analyzed with the help of gas chromatography.

For safety evaluation of the dermal exposure of consumers to azo dyes from wearing colored textiles, a possible cleavage of azo dyes by the skin microflora should be considered since, in contrast to many dyes, aromatic amines are easily absorbed by the skin. Azo dyes are metabolized to the corresponding amines by liver enzymes and the intestinal microflora following incorporation by both experimental animals and humans. The effects of skin cancer can be seen in photographs given below (Fig. 15.1).

The 22 amines used in azo dyes and reported by German scientists to be harmful are given in Table 15.1. The other dyes which have been reported to be toxic are given in Table 15.2.

A study was conducted by collecting the representative sample of colored handmade paper from

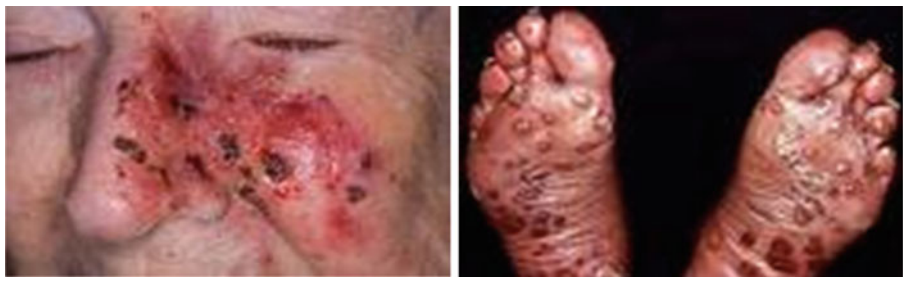


Fig. 15.1 Carcinogenic effect of azo dyes showing skin cancer

Table 15.1 List of harmful amines

4,4′-Methylene-bis-(2-chloroaniline)	3,3′-Dichlorobenzidine
2-Methoxyaniline	*p*-Aminoazobenzene
4-Aminodiphenyl	*p*-Cresidine
4-Chloro-o-toluidine	4,4′-Oxydianiline
2-Naphthylamine	3,3′-Dimethylbenzidine
p-Chloroaniline	3,3′-Dimethyl-4,4′-diaminodiphenylmethane
2,4-Diaminoanisole	4,4′-Thiodianiline
4,4′-Diaminodiphenylmethane	*o*-Toluidine
2,4-Diaminotoluene	2,4,5-Trimethylaniline
o-Aminoazotoluene	2-Amino-4-nitrotoluene
Benzidine	3,3′-Dimethoxybenzidine

Table 15.2 Toxic dyes with their lethal dosage concentration

		LD_{50}	Irritation	Irritation
		Mg/kg	Skin (rabbit)	Eye (rabbit)
Basic blue 7	CI42595	100	–	+
Basic blue81	CI42598	205	–	+
Basic red12	CI48070	25	–	+
Basic violet16	CI48013	90	–	+
Basic yellow 21	CI48060	171	–	+

different handmade paper units and evaluated for the nature of dyes including presence of azo group. The results showed that all handmade paper samples have been dyed with direct dyes. It was found that a specific shade has got more possibility of having azo group in comparison to others.

History of Natural Dyes

The history of natural dyes is very interesting. The earlier written record of the use of natural dyes was found in China dated 2600 BC. Chemical tests of red fabrics in the tomb of King Tutankhamen in Egypt show the presence of alizarin, a pigment extracted from madder. Alexander the great had deceived the Persians into thinking that his army was wounded by sprinkling his soldiers with red dye, probably madder juice which contains the dye alizarin. The first fiber dyes were already used in prehistoric times after the last Ice Age around 1000 BC. They consisted of fugitive stains from berries, blossoms, barks, and roots.

Natural dyes are dyestuffs made from plants, minerals, and, in the case of cochineal, insects. Some of natural dyes are indigo (*Indigofera tinctoria* Linn.), Indian madder (*Rubia cordifolia*), henna (*Lawsonia inermis*), catechu (*Acacia catechu*), ratanjot (*Onosma echioides*), tesu (*Butea monosperma* Lam. Kuntze), myrobalan (*Terminalia chebula*), tamarind (*Tamarindus indica* Linn.), safed kikar (*Acacia leucophloea* wild), etc. No doubt, natural dyes in comparison to direct dyes are more trouble and take longer to prepare. Some of the chemical colors (direct dyes) are not fast to light and washing, but natural dyes as madder, lichens, catechu, etc., are fast. Natural dyes have limited range of shades in comparison to synthetic dyes, so a lot of study is needed on evaluation of maximum possible shades.

Dyes and Their Source

Natural Dyes Obtained from Plants

Indigofera tinctoria

It is a substantive dye, which is different from any other dye as it does not require any mordant, yet the color achieved is extremely fast to washing and to light. Rather, it is dyed through a living fermentation process. Slowly the air

Table 15.3 Natural dyes

S. no.	Botanical name	Local name	Source
1.	*Punica granatum* Linn.	Anar	Rind of pomegranate
2.	*Indigofera tinctoria* Linn.	Indigo	Leaves
3.	*Terminalia chebula*	Harda	Fruit
4.	*Acacia catechu*	Cutch, Khayer, Katha	Bark
5.	*Terminalia arjuna*	Arjun	Bark
6.	*Kerria lacca*	Lac	Secretion of *Kerria lacca* on host plant palas
7.	*Rubia cordifolia* Linn.	Indian madder	Root of madder
		Manjishtha	
8.	*Eucalyptus camaldulensis*	Eucalyptus	Leaves
9.	*Lantana*	Lantana dye	Leaves
10.	*Lawsonia inermis* Linn.	Henna	Leaves
11.	*Butea monosperma* (Lam) Kuntze	Tesu flower	Flower
12.	*Pterocarpus santalinus* Linn. f.	Chandan	Bark of chandan
13.	*Acacia arabica*	Babul	Bark of babul

changes it to the beautiful deep and rich blue of indigo, which is having good light fastness. The leaves of *Indigofera tinctoria* plant are fermented in fermentation pits. The dye is precipitated by oxidation or by passing the air. The precipitated blue natural dye at the bottom is dried in the air to make the cake. The appearance of dye is blue, and the main coloring compound is indigotin.

Terminalia chebula

The dried fruits of *Terminalia chebula* (myrobalan) are used to produce the dye. The appearance of dye powder is brown, and the main coloring component is chebulinic acid. It is used in Ayurvedic preparations and has therapeutic value.

Quercus infectoria

The gallnuts of *Quercus infectoria* are pulverized to required particle size and extracted in aqueous media. The extract is filtered and sprayed in vacuum dryer to obtain the dye. *Q. infectoria* is a dried secretion of an insect living on the tree. The main coloring component is gallotannic acids, and appearance of dye powder is brownish yellow.

Acacia catechu

The cutch is used as raw material for manufacturing the dye, which is obtained from the leftover of katha produced from *Acacia catechu*. Katha is used as edible paste in pan preparations. The main coloring component is catechin/flavonoid.

Rubia cordifolia

It is contained in the roots of the madder plant. The plants are dug up, the roots are washed, dried, and ground into powder. It is found beneath the outer bark of the roots when the plants are about 3 years old. At this age, the roots of the plant render a suitable dye. Only about two percent dye is obtained from the dried roots. The madder named as "Turkey red" was very popular during the nineteenth century.

Terminalia arjuna

The bark and dried fruits of *Terminalia arjuna* are used for manufacturing the dye. The dry bark from the stem contains 15–24% tannin and is used in tanneries.

Tectona grandis

The dye is produced from dried leaves of teak tree. The main coloring compound in the dye is Tectoleafquinone.

A number of natural dyes obtained from plants is listed in Table 15.3 and photograph of such plants have been shown in Fig. 15.2.

Natural Dyes Obtained from Animals

Cochineal

It is an important red element for the natural dye user. It is the brightest of all the available natural red dyes. This dye is found in the cochineal louse,

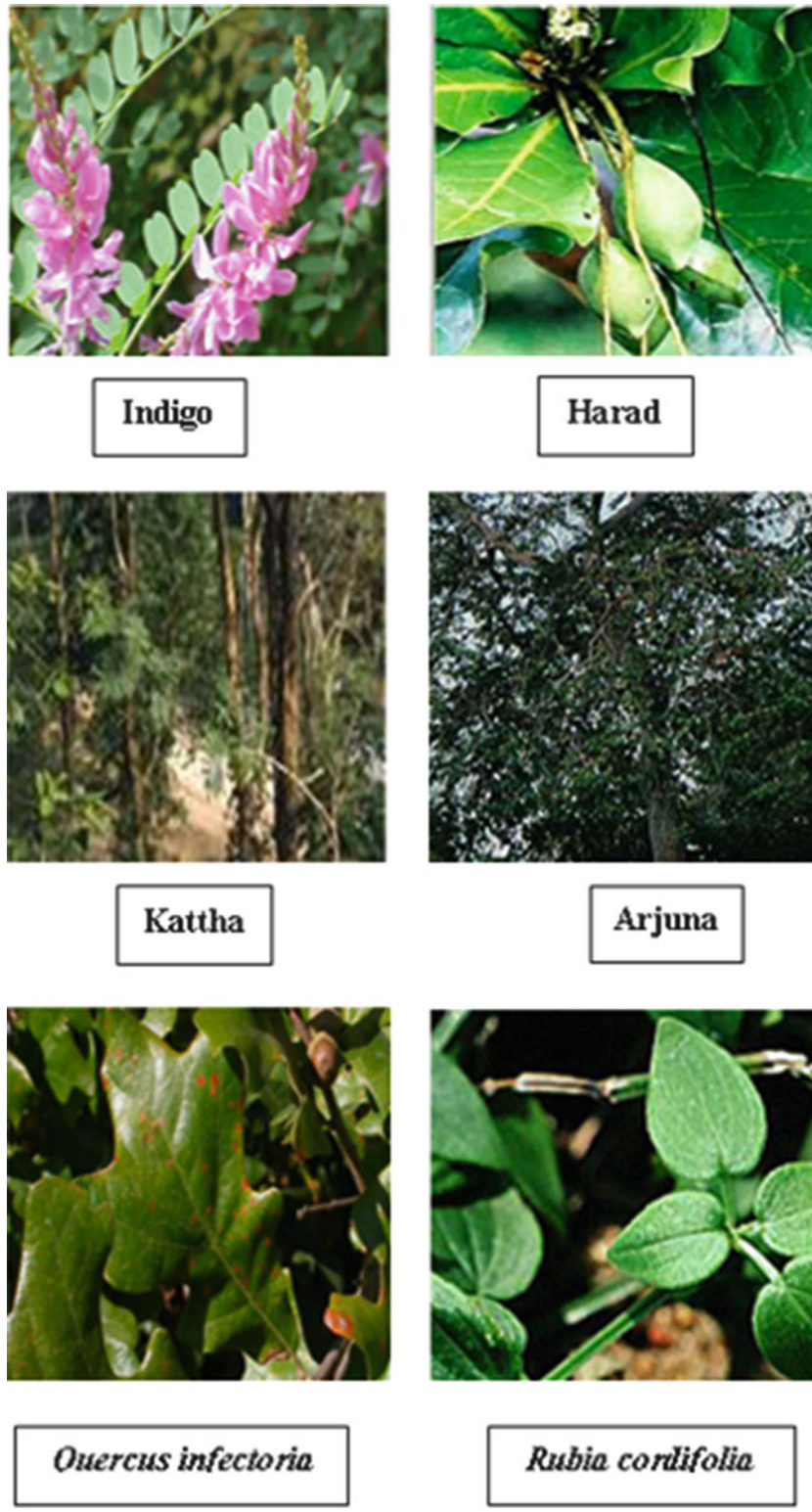


Fig. 15.2 Dye producing plants

which feeds on cactus plants. The female insect is dried in the sun and grounded to produce a rich red powder. Figure 15.3 shows the secretion of cochineal insect on cactus plant. The yield under the best conditions is about 2.72 kg per acre of cacti.

Kerria lacca

The dye is obtained as a byproduct of the secretion of insect *Kerria lacca*. The main coloring component is Laccaic acid.

Fig. 15.3 Secretion of cochineal insect on cactus plant

Natural Dyes Obtained from Minerals

Cinnabar (Hindi: Sangarf)
Red lead (Sindoor)
Red ochre (Geru)
Ultramarine (Lajwad)
Zinc white (Safeda)

Mineral dyes are obtained from an impure earthy ore of iron or ferruginous clay, usually red or yellow. Some of the most important mineral pigments used in India as analyzed from paintings on walls, cloth, and paper have been cinnabar (Hindi: Sangarf), red lead (Sindoor), red ochre (Geru), ultramarine (Lajwad), zinc white (Safeda), and yellow ochre (Ram Raj).

Yield of Natural Dyes

Yield of some of the natural dyes are as given in Table 15.4, while there characterization have been mentioned in Table 15.5.

Classification of Natural Dyes

First classification of natural dyes was according to alphabetical order. Later classification based on chemical structure, where grouping within

Table 15.4 Yield of natural dyes

S. no.	Dye	Source	Dye content (%)
1.	Madder	Root bark	1.9
2.	Cochineal	Female cochineal louse	1.8
3.	Indigo	Indigo plant leaves	1.5–2.0
4.	Saffron	Crocus plant flower pistil	7.0
5.	Annatto	Roucone-tree seed	15.0
6.	Carotin	Carrot, red palm oil, pumpkin seed	<0.5
7.	Lac	Stick lac	0.5–0.75

Table 15.5 Characteristics of some purified natural dye powders

Botanical name	Common name	Moisture	Water-soluble matter (%)	pH of 1% solution	Ash (%)
Acacia catechu	Cutch	6.0	95.0	6.0	7.0
Rubia cordifolia	Indian madder	5.0	95.0	8.0	35.0
Terminalia chebula	Myrobalan	5.0	97.0	3.5	7.0
Indigofera tinctoria	Indigo	5.0	4.0	5.0	63.0

each structure class is according to hue, was carried out. In color index, the dyes were classified according to chemical composition as well as major application classes. The natural color and hue of a dye can be altered by treating with metal salts. The color of dye originating from a plant depends on soil properties, part of plant, and season of harvesting, cultivation practices, etc.

Chemical Class

The natural organic dyes and pigments cover a wide range of chemical classes, namely, ketone, anthroquinoids, naphthoquinones, flavones, indigoids, and chlorophyll.

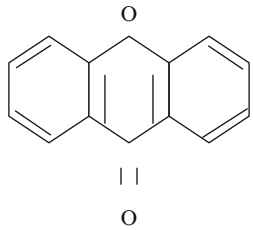
Indigoid Dyes

Indigo perhaps the oldest natural dye used by man occurs as the glucoside indicant in the plant *Indigofera tinctoria*. It is an example of vat dye.

Anthraquinone-Based Dyes

Some of the most important red dyes are based on the anthraquinone structure. They are obtained both from plants and insects or animals. These dyes are characterized by good fastness to light. They form complexes with metal salts, and the resultant metal complex dyes have good wash fastness.

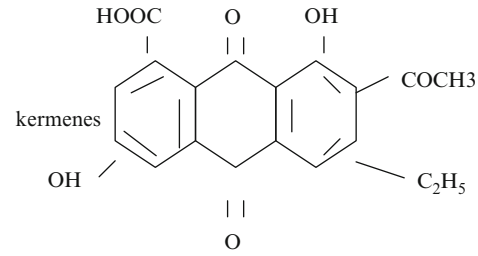
Madder dyes are hydroxyanthraquinones which are extracted from the root bark of various Rubiaceae, e.g., from madder root (*Rubia tinctorum*). The roots contain approximately 1.9% of dye present in the free form or bound as the glucoside. Madder also known as alizarin was the first natural dye to be synthesized chemically in 1869.



Anthra-quinonne

Lac Dye

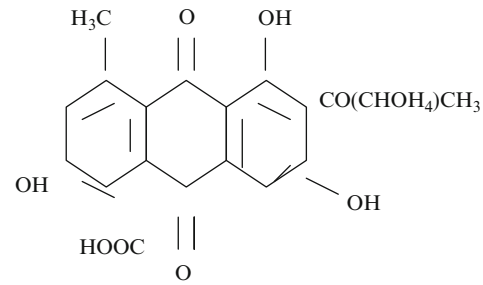
Lac is probably the most ancient of the animal dyes. It has been used in Southeast Asia and India since the beginning of the recorded history. The dye yields scarlet and crimson shades which exhibit good properties, especially to light and washing. Structurally, the principal coloring component has been identified as laccaic acid.



Laccaic acid

Cochineal

Cochineal is obtained from an insect of the same name which feeds on the cactus plant. Chemically cochineal is similar to kermenes. The main coloring component of this dye is carminic acid.



Carminic acid

Alpha-Naphthoquinones

The most prominent member of this class of dyes is *Lawsonia inermis* or henna. It is obtained from the leaves of *L. inermis*, cultivated mainly in India and Egypt. Lawsone has been identified as 2-hydroxy-1-4-naphthoquinone. Another similar dye is Juglone, 5-hdroxy-1-4-naphthoquinone obtained from the shells of unripe walnuts.

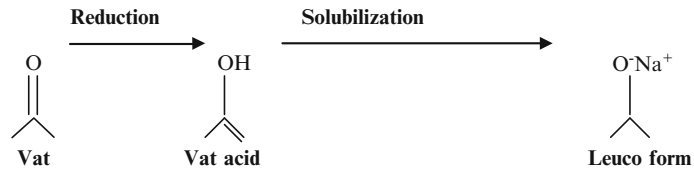


Fig. 15.4 Conversion of vat dyes to Leuco form

Application of Classes of Natural Dyes

Natural dyes were classified in two groups, namely, substantive and adjective dyes by Bancroft in his "Treatise on Permanent Colors." According to this classification, the dyes such as indigo, orchid, and turmeric, which dye the fibers directly, are classified as substantive dyes, while adjective dyes only dye material mordant with metallic salt; examples of such dyes are logwood, madder, cochineal, fustic, etc. In pure state the adjective dyes are generally slightly colored, and when used alone, they give poor dyeing.

Mordant Dyes

Dyes having affinity for the mordanted fibers or which form a complex with the mordant are called mordant dyes. The complex may be formed by first applying the mordant or by simultaneous application of the mordant and the dye or by after treatment of the dyed material. For a dye to be called as mordant dye, it should have electron-donating groups capable of forming complex with the transition metal salts. Alizarin was perhaps the first mordant dye. This dye has no affinity for cotton; however, on mordanting with a metal salt such as alum, it is readily absorbed by the fiber.

Vat Dyes

Vat dyes derive their name from the wooden fermentation vessel called the vat which was at one time used for reducing the dye and converting it into soluble form. The process of solubilization of the dye is known as vatting. The reaction of vat dyes to form Leuco form is shown in Fig. 15.4.

The soluble form of the dye is called leuco dye. The leuco form of the dye not only is soluble in water but has affinity for the natural fibers. The vatting of indigo can also be carried out by treatment with a reducing agent, such as sodium hydrosulfite and an alkali such as sodium hydroxide. The leuco or the reduced form of the dye is oxidized back to the original form on keeping the dyed material for 15–20 min at room temperature before washing. The true color is produced on treatment with hot soap solution.

Experimental Work and Results

The samples of commercially available natural dyes were procured. Application of natural dyes using cotton rags as raw material has been explored. The conventional dye, i.e., direct dye, was compared with natural dye to explore suitability of natural dyes in handmade paper sector. Pulp of cotton rags beaten up to 300-ml freeness level and dye of 1% concentration was prepared for the study.

Evaluation of Spent Liquor

The waste-spent liquor of natural dyes and direct dyes is evaluated for BOD (biochemical oxygen demand) and COD (chemical oxygen demand) which is determined with titration method. It has been found that BOD and COD of the effluent of natural dyes are far lower than that of direct dyes, as given in Table 15.6.

The above data clearly indicates the remarkable difference in COD and BOD values of effluent of dyes utilizing direct dyes and natural dyes. COD value of effluent of natural dye is approximately 65% lower than that of effluent of direct dyes, while BOD showed approximately 55% lower value of effluent of natural dyes in comparison to effluent of direct dyes.

Table 15.6 Comparison of effluent parameters of spent liquor of natural and direct dyes

Particulars	Natural dye (1%)	Direct dye (1%)
COD, ppm	800	2,400
BOD, ppm	110	250

Table 15.7 Light fastness of natural and direct dyed paper

S. no.	Dye and the form of paper	Light fastness
1.	*Quercus infectoria* (natural dye)	Scale 4
2.	*Quercus infectoria*, mordanted (natural dye)	Scale 4
3.	*Quercus infectoria*, sized (natural dye)	Scale 4
4.	*Rubia cordifolia* (natural dye)	Scale 4
5.	Pink, direct dye (synthetic dye)	Scale 2
6.	Pink, direct dye sized (synthetic dye)	Scale 2

Evaluation of Light Fastness of Paper

The light fastness of paper dyed with natural and synthetic dyes was evaluated in terms of scale with the help of Xenon Arc tester by exposing the sample for 24 h. The results of the same are given in Table 15.7.

The higher the value of scale, the better the light fastness. Natural dyes showed twice better light fastness in comparison to direct dyes with the help of Xenon Arc tester.

Comparison of Synthetic Dyes and Natural Dyes

No doubt, natural dyes in comparison to direct dyes are more trouble and take longer to prepare. Direct dyes are not fast to light and washing, but natural dyes are having very good fastness to light. When direct dyes fade, it becomes a different color and generally a bad one, while when a natural color fades, it becomes a lighter tone of the same color. Natural dyes are extracts from therapeutic plants (madder, indigo, Brazil wood, logwood, fustic, catechu/cutch), insects (cochineal), and naturally occurring minerals (alum, iron, tin) needed by body in trace amounts. Natural dyes have limited range of shades in comparison to synthetic dyes, so a lot of study is needed on evaluation of maximum possible shades.

Conclusion

The study conducted on application of natural dyes in handmade papermaking nullifies the possibility of paper having azo group, thus enhancing the eco-friendly credentials of handmade papermaking. This will boost export of Indian handmade paper in international market. Use of natural dyes in colored handmade paper needs to be propagated to eliminate the possibility of presence of toxic azo group in the paper due to synthetic dyes. This would help in retaining the eco-friendly credentials of handmade paper.

References

Cheryl Kolander (1995) Master Natural Dyer, Aurora Silk

Kolander C (2004) "Brilliant colours with natural dyes on silk and other natural textiles" Master natural dyer Aurora Silk, 1st edn. Mama, D.O.C, Portland

Mairet EM, Pepler D (1916) A book on vegetable dyes. Published by Douglas Pepler at Hampshire House, Workshops, Hammersmith

Saakshy AKS (2006) Application of natural dyes in handmade paper making. Souvenir Indian Paper 2006, Vapi, 10–13 Nov 2006

Textile Committee (2001) Textile Committee report, Mumbai, Oct 2001

Patenting Trends in Bioremediation Technologies for Oil-Contaminated Sites

16

Rajeev Kumar Kapoor, Rishi Gupta, and Ajay Singh

Abstract

Controlling pollution caused by hydrocarbon compounds, for example, crude oil pollution, in soils and waters by bioremediation has acquired considerable significance. Bioremediation is one technique that may be useful to remove spilled oil under certain geographic and climatic conditions. Researchers all around the world have developed different methodologies to reclaim the contaminated site according to the prevailing environmental conditions. A set of 125 patent/patent applications describing the use of oil-degrading microorganisms were analysed to find out the approach followed to achieve bioremediation. Patent documents revealed that the technique of bioremediation using microorganism follows two broad approaches, viz. fertilization and seeding. Fertilization is the bioremediation method of adding nutrients, such as nitrogen and phosphorus, to a contaminated environment to stimulate the growth of indigenous microorganisms, and seeding refers to the addition of naturally occurring or genetically engineered microorganisms to a spill site. Overall the success of a technique depends on the ability to establish appropriate conditions for the microorganisms in the contaminated environment. Another approach in a set of patents showed that growth may be enhanced by increasing the substrate bioavailability or by emulsification. United States

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of America is the leading country followed by China, Korea, Japan, and Russia in developing bioremediation technologies. Prominent companies working towards developing eco-friendly solutions include DuPont, Biosaint, Univ Nat Cheng Kung, Samsung Everland Inc., Technology Licensing Organization, Biorem AG, Petrozyme Technologies, Ocean University of China, and BioNutraTech.

Keywords
Oil • Hydrocarbon • Bioremediation • Microorganism • Patent

Introduction

Biodegradation refers to the natural process whereby microorganisms alter and break down organic molecules into other substances, such as fatty acids and carbon dioxide, and bioremediation is the act of adding materials to contaminated environments, such as oil spill sites. Amongst the other applications for which bioremediation is being considered or is currently in use are (1) treatment of nontoxic liquid and solid wastes (Liu et al. 2011), (2) treatment of toxic or hazardous wastes (Atlas and Hazen 2011), (3) treatment of contaminated groundwater, and (4) grease decomposition. Although recent marine oil spills and bioremediation efforts have called attention to the potential of bioremediation as an oil spill response technology, some of these other applications, in particular the treatment of hazardous waste, appear to have greater potential (Lin et al. 2011; Galindo et al. 2011; Chandran and Das 2011). Officials at approximately 135 hazardous waste sites, for example, are now either considering, planning, or operating full-scale bioremediation systems. A large number of refineries, tank farms, and transfer stations now employ in situ bioremediation methods to restore land contaminated by accidental spills of fuel oil or other hydrocarbons (Zhang et al. 2011). Much less progress has been made with respect to the practical problems of applying bioremediation technologies to marine oil spills, although advocates have suggested their use in the wake of several major spills.

The problems associated with using bioremediation technologies in marine environments are fundamentally different from those associated with land-based applications. Potential bioremediation approaches for marine oil spills fall into three major categories:

(a) Stimulation of indigenous microorganisms through addition of nutrients (fertilization) (Chamkha et al. 2011; Mohite et al. 2011; Korotkevych et al. 2011)
(b) Introduction of special assemblages of naturally occurring oil-degrading microorganisms (seeding)
(c) Introduction of genetically engineered microorganisms (GEMs) with special oil-degrading properties

Stimulation of indigenous organisms by the addition of nutrients is the approach that has been tested most rigorously. This approach is viewed by many researchers as the most promising one for responding to most types of marine spills. Recent experiments suggest that rates of biodegradation in most marine environments are constrained by lack of nutrients rather than by the absence of oil-degrading microbes (Churchill et al. 1995). The introduction of microbes might be beneficial in areas where native organisms grow slowly or are unable to degrade a particular hydrocarbon. However, the effectiveness of this approach has not yet been demonstrated. The wide availability of naturally occurring microorganisms capable of degrading components of petroleum will likely deter consideration of GEMs for remediating marine oil spills.

Degradation of hydrocarbons in the presence of synthetic surfactants is a delicate issue. Generally, the toxicity of surfactants increases with their hydrophobicity (Tiehm 1994). The use of surfactants of biological origin solves the toxicity problem.

Moreover, greater research and development needs, regulatory hurdles, and public perception problems will remain obstacles to the near-term use of GEMS even if they could prove useful for degrading some recalcitrant components of petroleum. Bioremediation technologies for beach cleanup have so far received the most attention (MacNaughton et al. 1999; Lloyd-Jones et al. 1999; Mesarch et al. 2000). Experiments conducted by United States Environmental Protection Agency (EPA), Exxon, and the State of Alaska on cobble beaches fouled by oil from the Exxon Valdez indicated that the addition of nutrients at least doubled the natural rate of biodegradation. The efficacy of commercial microbial products in remediating beaches is not yet known. Limited EPA field tests using two microbial products on heavily weathered oil in Alaska were inconclusive. Additional field experiments are required on other types of beaches that involve different oils and different climatic and marine conditions. The objective of this chapter is to analyse the current state of development of bioremediation technology through the patent documents filed in all the jurisdiction of this world. This chapter also describes some exemplary techniques used for the bioremediation of oil-contaminated sites and to clean up marine oil spills.

Methods

The scope of the study was to identify commercially viable patent families describing different bioremediation strategies to overcome pollution caused by oils. To shortlist the potentially relevant patent families in the field of bioremediation, search strings were made using the keywords based on the understanding developed for conducting a search for patents/published applications in the field of study.

Patents and published patent applications were extracted from Thomson Innovation patent database.

Results

Running search string S-1 in Thomson Innovation patent database yielded 390 patents and published applications. The patents and published applications obtained from Thomson Innovation patent database were reduced to 'one member per patent family'. A unique set of 248 distinct patent families was obtained. A title-, abstract-, and claim-based screening was carried out to select certain technologies which were describing the use of microorganisms for bioremediation. One hundred and twenty five patent documents were short listed and analysed in more details.

Most Frequently Used Microbial Groups

Analysis of the 125 patent documents revealed that more than 100 different genera of microorganisms have been claimed for oil degradation; major genera amongst these include (in decreasing order) *Pseudomonas, Rhodococcus, Bacillus, Thauera, Acinetobacter, Shewanella, Stenotrophomonas, Gordonia, Burkholderia, Alcaligenes, Pseudomonas, Marinobacter, Azotobacter, Comamonas, Marino-bacterium, Petrotoga, Microbulbifer, Alcanivorax, Yarrowia, Flavobacterium,* and *Moraxella* (Table 16.1).

Representative Technologies

Complete analysis of the patent documents showed the following basis of achieving bioremediation of an oil-contaminated site:

1. Use of an enriched steady-state microbial consortiums.
2. Oil is broken down into microscopically small parts by way of some novel composition.
3. Use of a chemical mixture which enhances the substrate bioavailability for micro-organism.

Table 16.1 Microorganisms claimed for degradation of oil in different patent documents filed by their respective assignees

Publication number	Title of patent	Microorganisms claimed	Priority date – earliest	Assignee/applicant	Priority country
US6649400B2	Bacteria mixture having heavy oil degrading ability and method of treating oil components	*Acinetobacter bacteria; Alcaligenes bacteria; Flavobacterium bacteria; Moraxella bacteria*	29-03-1999	Technology Licensing Organization Inc., Osaka, JP	Japan
US5342525A	Method for aiding microbial degradation of spilled oil	*Bacillus subtilis; Pseudomonas aeruginosa; Azobacter species, Xanthomonas species*	28-10-1991	Rowsell Farrell D	United States of America
US20100051541A1	Bioremediation method for accelerated biological decomposition of petroleum hydrocarbons in sea ice-covered polar regions, and bacteria and enzyme mixtures as agents for carrying out said method	*Rhodococcus GH-1; Dietzia GH-2; Shewanella GH-4; Marinobacter GH-9; Pseudomonas GH-10; Oleispira GH-11; Marinobacter GH-3; Marinomonas GH-5; Pseudoalteromonas GH-6; Psychrobacter GH-7; Jannaschia GH-8*	21-01-2007	Stiftung Alfred-Wegener-Institut Fuer Polar-Und Meeresforschung, Bremerhaven, de	Germany
EP1132462A1	Bacteria strains having heavy Oil degrading ability, mixtures thereof and nurturing composition therefore	*Strain FERMBP-7046 belonging to the genus Acinetobacter, a strain FERMBP-7049 belonging to the genus Acinetobacter, a strain FERMBP-7047 belonging to the genus Pseudomonas, and a strain FERMBP-7048 belonging to the genus Alcaligenes*	09-03-2000	Technology Licensing Organization Inc., Osaka, JP	European Patent Office
US5575998A	Mixture of microorganisms, its use for the biodegradation of hydrocarbons, as well as process for its application	*Pseudomonas putida and Geotrichum candidum*	15-04-1992	Biorem AG,ZUG,CH	Czechoslovakia (CZ or SK after 1992)
US7083968B2	Microorganism of Serratia family, isolation method and t he preparation method of lignin lyases using this	*Serratia marcescens HY-5*	20-06-2001	Insect Biotech Co. Ltd.,KR	Korea (South)

US20100209988A1	Bioremediation materials	*Arthrobacter, Actinobacter, Pseudomonas spp., Mycobacteria, Rhodococcus spp., Sphingomonas,* fungi that produce peroxides and laccases, *Bacillus spp., Pseudomonas fluorescens, mycorrhiza, Rhizobium spp., Frankia, Azotobacter, Anabaena, Nostoc, Beijerinckia, Klebsiella, Lactobacilli, Bifidobacterium, Enterococcus faecium, Lactococcus lactis, Leuconstoc mesenteroides, Pediococcus acidilactici, Streptococcus thermophilus, Bacillus subtilis, Escherichia coli strain Nissle, Saccharomyces boulardii, and Saccharomyces cerevisiae*	08-11-2005	The University of Surrey the Forrestry Commission	Great Britain
US20050227338A1	Production of biomass and single cell protein from industrial waste oils, oily sludge from ships and other sources of oily wastes	*Pseudomonas aeruginosa, Phenylobacterium immobile, Stenotrophomonas maltophilia, Gluconobacter cerinus, Agrobacterium radiobacter, and Citrobacter freundii and other indigenously available species*	25-03-2004		United States of America
US7708065B2	Identification, characterization, and application of Thauera Sp. Al9:8 useful in microbially enhanced oil recovery	*Thauera strain AL9:8 (ATCC No. PTA 9497), Pseudomonas stutzeri LH4:15 (ATCC No. PTA-8823)*	29-09-2008	E.I. du Pont de Nemours and Company, Wilmington, DE, US	United States of America
KR2011117358A	Novel microorganism having oil degradation ability and a method for biologically recovering oil-polluted soil using the same	*Pseudomonas sp. MJ32(KCTC11678BP) and Pseudomonas sp. MJ33(KCTC11679BP)*	21-04-2010	H-Plus Eco Ltd.	Korea (South)
US20110030956A1	Altering the interface of hydrocarbon-coated surfaces	*Shewanella, Pseudomonas stutzeri LH4:15 (ATCC No. PTA-8823), Thauera sp. AL9:8, (ATCC No. PTA-9497)*	22-05-2009	E.I. du Pont de Nemours and Company, Wilmington, DE, US	United States of America

(continued)

Table 16.1 (continued)

Publication number	Title of patent	Microorganisms claimed	Priority date – earliest	Assignee/applicant	Priority country
US20080020947A1	Novel microorganisms having oil biodegradability and method for bioremediation of oil-contaminated soil	*Rhodococcus baikonurensis, Acinetobacter johnsonii, Acinetobacter haemolyticus, Nocardia transvalensis, Nocardia asteroides, Gordonia sputi, Gordonia rhizosphera, Gordonia nitida, Gordonia hirsuta, Gordonia bronchialis, Gordonia amarae, Gordonia desulfuricans, Rhodococcus zopfii, Rhodococcus wratislaviensis, Rhodococcus tukisamuensis, Rhodococcus ruber, Rhodococcus rhodochrous, Rhodococcus rhodnii, Rhodococcus pyridinovorans, Rhodococcus percolatus, Rhodococcus opacus, Rhodococcus marinonascens, Rhodococcus koreensis, Rhodococcus jostii, Rhodococcus globerulus, Rhodococcus fascians, Rhodococcus erythropolis, Rhodococcus erythreus, Rhodococcus equi, Rhodococcus coprophilus, Rhodococcus baikonurensis, Acinetobacter towneri, Acinetobacter baylyi, Acinetobacter calcoaceticus, Acinetobacter grimontii, Acinetobacter lwoffii, Acinetobacter radioresistens, Acinetobacter tandoii, Acinetobacter baumannii, Acinetobacter bouvetii, Acinetobacter gerneri, Acinetobacter junii, Acinetobacter parvus, Acinetobacter schindleri, Acinetobacter*	18-07-2006		Korea (South)
WO2002004364A2	Process for biodegrading crude oil	*Comanonas acidovorans, Megateria, Acinetobacter, Arthrobacter, Rhodococcus, Pseudomonas aeruginosa, and Pseudomonas putida*	07-07-2000	BP Exploration Operating Company Limited, GB	Great Britain
JP2000083651A	Microorganism capable of degrading heavy oil	*Caulobacter FERM P-16978*	09-09-1998	Shikoku Instrumentation Co Ltd.	Japan
US20120006540A1	Method for pre-treatment of subterranean sites adjacent to water injection wells	*Comamonas, Fusibacter, Marinobacterium, Petrotoga, Shewanella, Pseudomonas, Vibrio, Petrotoga, Thauera, and Microbulbifer*	09-07-2010	E.I. du Pont de Nemours and Company, Wilmington, DE, US	United States of America

KR2005043506A	Microorganisms capable of removing contaminants by effectively degrading contaminants in soil contaminated by oil, activator capable of increasing degradation activity of the microorganisms, and method for remediation of soil contaminated by oil using the microorganisms and activator	*Rhodococcus sp., Acinetobacter sp., Gordonia sp., and a mixed cultured strain*	06-11-2003	NEO Pharm Co. Ltd.	Korea (South)
KR2002060009A	Novel microorganism for the degradation of crude oil and process for production thereof	*Pseudomonas sp. CU1(KCTC 18065P)*	09-01-2001	ECO Solutions Co. Ltd.	Korea (South)
DE4014854A1	Verfahren Und Zugehoerige Vorrichtung Zum Entfernen Von Oel- Und Fettablagerungen Auf Beton-, Zement- Oder Steinboeden	*Pseudomonas putida, Thiobacillus thiooxidans, and Thiobacillus ferrooxidans*	09-05-1990	Biek Volker DR.,DE,0041556530DE	Germany
US20120006541A1	Method for pre-treatment of subterranean sites adjacent to water injection wells	*Pseudomonas, Corynebacteria, Achromobacter, Acinetobacter, Arthrobacter, Bacillus, Nocardia, and Vibrio*	09-07-2010	E.I. du Pont de Nemours and Company, Wilmington,DE, US	United States of America
KR2000034035A	Bioaugmentation of oil contaminated soil by microbial composition which can decompose hydrocarbons derived from petroleum	*Acinetobacter sp. W1, Pseudomonas sp. HB26, and Bacillus sp. L11*	27-11-1998	In Bionet. Inc.	Korea (South)
CN101781624B	Bacterial strain used for produced water treatment of oil field	*Candida viswanathii*	29-12-2009	Petrochina Co Ltd	China
CN101531974A	Arctic bacteria strain for highly efficiently degrading crude oil and application thereof	*P29 belonging to Gram-negative Pseudoalteromonas sp.*	21-03-2009	First Ocean Research Institute National Bureau of Oceanography,	China

(continued)

Table 16.1 (continued)

Publication number	Title of patent	Microorganisms claimed	Priority date – earliest	Assignee/applicant	Priority country
CN101486980A	Solid microbial preparation for petroleum pollutant and oil product degradation, preparation and use	*Pseudomonas aeruginosa*	16-02-2009	Yu Yang, Jinan, Shandong 250014,CN	China
LV14133B	Isolation of consortium of microorganisms-destructors Mdk-825 for oil product degradation in soil	*MDK-825 for this microorganism consortium*	16-10-2008	Latvijas UNI	Latvia
CN101948786A	*Pseudomonas aeruginosa* for producing rhamnolipid with high yield and application thereof	*Pseudomonas aeruginosa*	03-09-2010	Petrochina Co Ltd	China
WO2012050794A1	Control of fluid flow during treatment of subterranean sites using well fluid injection	*Pseudomonas, Bacillus, Actinomycetes,Acinetobacter, Arthrobacter, Schizomycetes, Corynebacteria,Achromobacteria, Arcobacter, Enterobacteria, Nocardia, Saccharomycetes, Schizosaccharomyces, Vibrio, Shewanella, Thauera, Petrotoga, Microbulbifer, Marinobacteria, Fusobacteria, and Rhodotorula*	29-09-2010	E.I. du Pont de Nemours and Company,Wilmington,DE, US	United States of America
CN101691552A	Bacterial strain capable of degrading petroleum hydrocarbon and application thereof	*Citrobacter species WTS (CGMCC Number 3253)*	15-09-2009	Peking University, Beijing 100871,CN	China
US20100216219A1	Method of in situ bioremediation of hydrocarbon-contaminated sites using an enriched anaerobic steady state microbial consortium	*Rhodocyclaceae, Pseudomonadales, Bacteroidaceae, Clostridiaceae, Incertae sedis, Spirochaetaceae, Deferribacterales, Brucellaceae, and Chloroflexaceae; Marinobacterium georgiense, Thauera aromatica T1, Thauera chlorobenzoica, Petrotoga miotherma, Shewanella putrefaciens, Thauera aromatica S100, Comamonas terrigena, Microbulbifer hydrolyticus (ATCC#700072)*	23-02-2009	E.I. du Pont de Nemours and Company, Wilmington, DE, US	United States of America

RU2272071C2	*Saccharomyces* Sp. and *Pseudomonas* Sp. strain microorganisms, uses thereof as decomposers in bioremediation of oil-contaminated environmental Objects and associations based on the same	*Saccharomyces sp. and Pseudomonas sp.*	10-03-2004		Russia (SU before 1993)
JP2003116526A	Highly functional fatty oil-decomposing bacterium and method of bioremediation using the same	*Pseudomonas*	12-10-2001	Japan Science & Technology Corp	Japan
JP11075832A	Microorganism mixture and degradation of oil and fat	*Chromatium buderi, Amoebobacter roseus, Rhodopseudomonas acidophila, and Rhodopseudomonas capsula*	05-09-1997	S K T Kenkyusho KK | Nakano Akira	Japan
KR2011009403A	Functional microorganism community with an activity of decomposing marine crude oil	*Alcanivorax sp., Idiomarina sp., Oleiphilus sp., and Sphingomonas sp.*	22-07-2009	Korea Research Institute of Bioscience and Biotechnology	Korea (South)
KR938761B1	Pseudomonas Sp. Dr2 producing biodegradable plastic using waste vegetable oil and the method for producing polyhydroxyalkanoate	*Pseudomonas species*	16-11-2007	Korea University Research and Business Foundation	Korea (South)
KR2003076142A	Hydrocarbon-degrading psychrotrophic microorganism *Rhodococcus* sp. Yhlt-2 Kctc 10203Bp strain and method for bioremediation of oil using the same	*Rhodococcus sp. YHLT-2 KCTC 10203BP*	19-03-2002	Biosaint Co. Ltd.	Korea (South)
KR2002015567A	*Stenotrophomonas maltophilia* T3-C, and method for bioremediation of oil contamination using the strain	*Stenotrophomonas maltophilia*	22-08-2000	Biosaint Co. Ltd.	Korea (South)
CN101186890B	Diesel oil alkane component degradation bacterium and application thereof	*Burkholderia cepacia*	14-12-2007	Univ South China Tech	China

(continued)

Table 16.1 (continued)

Publication number	Title of patent	Microorganisms claimed	Priority date – earliest	Assignee/applicant	Priority country
GB2342303A	Method and apparatus for removing oil from oil-contaminated particulate material as E.G. Waste drilling mud	*Rhodococcus or Gordona or Tsukamurella*	24-07-1997	The Court of Napier University	Great Britain
EP846084A1	Use of the yeast *Yarrowia lipolytica* W1 for the industrial biodegradation of oils and fats in sewage and biodegradation process using it	*Yarrowia lipolytica*	29-05-1995	Biorem AG, ZUG, CH	Czech Republic (CS before 1993)
RU2180276C1	Oleophilic biological preparation useful for cleaning oil-polluted soil	*Rhodococcus erythropolis IEGM 708 and Rhodococcus rubber IEGM 327*	19-02-2001	Inst Ehkologii I Genetiki Mikr	Russia (SU before 1993)
RU2344170C2	Strain of bacteria *Pseudomonas putida* producing surface-active substances for polycyclic aromatic hydrocarbon and oil hydrocarbon degradation	*Pseudomonas putida ' -2380'*	10-03-2006	Filonov Andrej Evgen Evich	Russia (SU before 1993)
US20090148881A1	*Geobacillus thermodenitrificans* as well as the screening method and the uses thereof	*Geobacillus thermodenitrificans*	17-11-2004	Nankai University, Tianjin, CN	China
CN101225366B	*Minimal gingivalis* T7-7 degrading hydrocarbon under low temperature and uses thereof	*Pusillimonas sp. T7-7*	24-09-2007	Univ Nankai	China
KR2006089001A	Microorganism *Yarrowia* Sp. capable of degrading lubricating oil and other oils and use thereof for purifying area Polluted with lubricating oil and other oils	*Yarrowia sp.*	03-02-2005	National Institute of Environmental Research	Korea (South)
KR2001103140A	*Pseudomonas* Sp. Hplc-1 degrading crude oil	*Pseudomonas sp.*	31-12-1999	Samsung Everland Inc.	Korea (South)
US20100216217A1	Steady state anaerobic denitrifying consortium for application in in-situ bioremediation of hydrocarbon-contaminated sites and enhanced oil recovery	*Thauera strain, Azoarcus species, Pseudomonas species, Azotobacter species, Bacteroides species, Clostridium species, Anaerovorax species, Finegoldia species, Spirochetes species, Deferribacter species, Flexistipes species, Chloroflexi species, and Ochrobactrum species*	23-02-2009	E.I. du Pont de Nemours and Company, Wilmington, DE, US	United States of America

CN101717725A	Microbial inoculum for bioremediating coastline polluted by oil spilling and preparation method thereof	*Bacillus pumilus CGMCCnO.3384 fermentation liquor, Venice acinetobacter CGMCCnO.3385 fermentation liquor and/or Doxa pseudomonad CGMCCnO.3386 fermentation liquor and/or Ralstonia pickettii CGMCCnO.3387*	25-12-2009	China National Offshore Oil Corporation, Beijing 100010, CN	China
CN101974466A	*Gordonia alkanivorans* and application in oil degradation	*Gordonia alkanivorans S104*	28-10-2010	Dalian University of Technology, CN	China
RU2199406C2	Method of cleaning grounds from oil pollution	*Bacillus sp. VNIISKhM 132*	25-04-2001	Inst Biolog Ufim Nauchnogo Tse	Russia (SU before 1993)
JP2001037466A	Biodegradation of heavy oil	*Alcanivorax such as GR211-P1 strain (FERM P-17394) or GR211-P2 strain (FERM P-17395) and the genus Bacillus such as GR211-W strain (FERM P-17396) or GR211-Y strain (FERM P-17397)*	24-05-1999	Agency of IND Science &	Japan
JP2001061468A	Oil-and-fat-degradative *Bacillus* and effluent treatment process using the same	*Bacillus subtilis (FERM P-17512)*	25-08-1999	Imanaka Tadayuki | Saito Masaaki	Japan
CN101935630A	*Rhodococcus* strain and use thereof in petroleum microorganism yield increase	*Rhodococcus equi*	02-07-2010	Dalian Bit Life Sciences Co. Ltd., CN	China
CN101914470A	*Acinetobacter calcoaceticus* and culture method and application thereof	*Acinetobacter calcoaceticus WG-6*	22-07-2010	Shandong Academy of Sciences Inst. of Biology, CN |	China
CN101935631A	*Ralstonia* sp. and application thereof in bioremediation of petroleum-contaminated saline-alkali soil	*Ralstonia strain XB*	02-07-2010	Binzhou University,CN	China
KR2002011251A	Biosurfactant-secreting bacteria and its utilization method	*Rhodococcus globerulus (KFCC-11171)*	01-08-2000	Samsung Everland Inc.	Korea (South)
TW200927922A	Novel *Pseudoburkholderia malthae* strain and their application in oil degradation	*Pseudoburkholderia malthae CC-AFH3.*	28-12-2007	Univ Nat Cheng Kung	Taiwan

(continued)

Table 16.1 (continued)

Publication number	Title of patent	Microorganisms claimed	Priority date – earliest	Assignee/applicant	Priority country
RU2237711C1	Method of restoring oil-polluted bleaching earth	*Bacillus brevis and Arthrobacter species*	30-12-2002		Russia (SU before 1993)
RU2297290C1	Method of recultivation of the bleaching soil polluted with the oil products	*Bacillus brevis IB DT 5–1 and Arthrobacter species IB DT 5–3 and the strain of bacteria Azotobacter vinelandii IB 4*	04-10-2005	NPP Biomedkhim Zao NPP Biomedk	Russia (SU before 1993)
TWI270535B	Novel *Rhodococcus erythropolis* and the purpose for oil degradation thereof	*Rhodococcus erythropolis CC-BC11*	02-04-2004	Univ Nat Cheng Kung	Taiwan
TWI270534B	Novel *Gordonia nitida* and the purpose for oil degradation thereof	*Gordonia nitida CC-JD39*	02-04-2004	Univ Nat Cheng Kung	Taiwan
KR2007020711A	Biological remover for removing oil pollutants, comprising peatmoss, *Bacillus* Sp. strain and/or *Pseudomonas* Sp. Strain, equipment for spraying the same, and remover pack comprising the same	*bacillus sp. strain and/or Pseudomonas sp.*	16-08-2005	Korea Paramount Co. Ltd.	Korea (South)
KR2003066948A	*Burkholderia cepacia* 2A-12 and method for bioremediation of Pah contamination using the strain	*Burkholderia cepacia 2A-12(KCTC 10163BP)*	06-02-2002	Biosaint Co. Ltd.	Korea (South)
CN1904033A	Diesel oil alkane eating bacteria and its degradation enzyme system	*Alcanivorax dieselolei NO1ACCTCC NO:M205024*	20-07-2005	National Ocean Bureau Third Ocean Institute,	China
JP2010178749A	Method for treating oil and fat-containing substance	*Cryptococcus laurentii*	19-03-2010	Fukushima Univ	Japan
KR837877B1	Functional microbial community, Fmc-Jw12 being capable of degrading diesel oil	*Functional microbial community FMC-JW12 comprises Sphingomonas sp., Aeromonas sp., Bacillus sp., Burkholderia sp., Propionibacterium sp., Stenotrophomonas sp., and Acinetobacter sp.*	23-03-2007	Korea Research Institute of Bioscience and Biotechnology	Korea (South)
JP1187085A	Novel lipase, production thereof and microorganisms producing same	*Alcaligenes f-B-24 (FERM-9715)*	18-01-1988	Osaka prefecture	Japan

4. Use of some particulate material for promoting growth of petroleum-degrading bacteria.
5. Use of a nutrient microemulsion in spray form, useful as a biodegradation accelerator.
6. Use of a nutrient mixture which has an emulsifying effect and would contain P- and N-yielding substances.
7. Use of a combined approach by using microorganisms directly and by promoting growth of oil-degrading bacteria.

Strategy 1

Use of Anaerobic Microorganisms Contained in Capsules, Coated Pellets, or Pods Having Cores of Varying Densities: The first strategy disclosed by Leon Kirschner (1999) in his European patent EP1088109B1 describes the use of an anaerobic microorganism *Vibrio desulfuricans* for remediation of submerged petroleum products, which anaerobes result in an overall exponential increase in speed of remediation, and also adds versatility to the art of remediation as applied to the inadvertent release of petroleum products into an aqueous environment during transport or storage, or to an intentional release of oil products, which also requires remediation.

The use of such anaerobic microorganisms offers bioremediation to those petroleum products which sink below the surface of the water and often to the floor of the sea, where known application techniques for remediation do not reach submerged oil spills.

Preemption is achieved by incorporating within the petroleum products, before a spill occurs, remediating agents contained in capsules, coated pellets, or pods having cores of varying densities. Such agents are inert and inactive when encapsulated or within the coated pellets, but are activated when not in the encapsulated environment. These encapsulated or pelletized agents are preferably dispersed throughout a body of petroleum. The capsules or pellets may be installed into the petroleum at the wellhead or in a pipeline, in a cargo hold, at a refinery, or at any desired location during the processing and transport or storage of the petroleum product.

Preferably, these capsules or pellets are reclaimable when not activated and may be reused. Such reclamation may be accomplished through magnetic (utilizing either ferromagnetic, diamagnetic, or paramagnetic materials, or a combination of some or all of them) separation, centrifugation, filtration, electrostatic or ionic precipitation, vaporization, or heat or vacuum distillation or a combination of such techniques. The capsules or pellets may be constructed to permit ease of reclamation and reuse.

Capsules or pellets with a core containing microorganisms, surrounded by time dissolvable layers of nutrients and other enhancements, may be compounded using prior art skills and formulations. Such capsules, pellets, or pods of varying sizes and/or densities, so as to embody the present invention, may also be novelly compounded to include iron or other compound [within either the capsule, pellet, or pod core and/or their surrounding (encapsulating) material or coating (pellet)] so as to permit the capsules, pellets, or pods to be varied in density, preferably to approximate the density of the petroleum product in which they are placed, and to encourage their removal from a body of petroleum by the techniques described above, when desired. The capsule, pellet, or pod may also be sprayed with an aqueous soluble electrostatically charged material or marked with material having magnetic, electrostatic, or electroconductive properties. The core or encapsulating material or coating may also be either fabricated with gas bubbles or contain evacuated or gas-filled glass beads (bubbles or beads of varying sizes) so as to affect the density of the capsule or pellet. These capsules or pellets may also contain water or other aqueous solutions to be time released so as to activate release of microorganisms when oil transport (e.g. pipeline) occurs under arid (e.g. desert) conditions to aid remediation of a petroleum spill or discharge into a nonaqueous environment.

Preferably, such capsules, pellets, or pods contain microorganisms, both aerobes and anaerobes or either of them.

With reference to the accompanying drawings and particularly to Figs. 16.1 and 16.2, a microcapsule cell 10 of anaerobic and aerobic microorganism

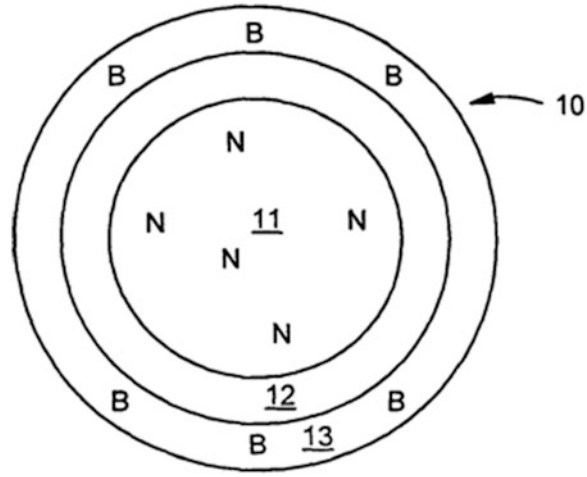

Fig. 16.1 Is a schematic view of a typical cell for the capsule, pellet, or pod described in the present invention

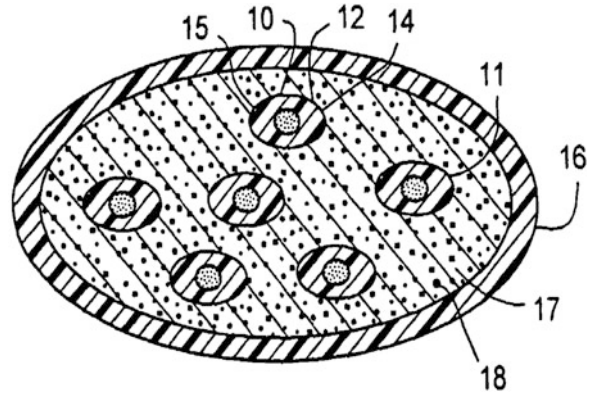

Fig. 16.2 Is a sectional view of a typical pod of the bioremediation product containing multiple cells

material having a microorganism nucleus (N) 11 is capable of degrading a hydrocarbon, which may comprise bacterium which degrades petrochemicals, such as organisms from the genus *Vibrio, Micrococcus, Bacillus, Arthrobacter, Nocardia, Corynebacterium and also Vibrio desulfuricans, Arthrobacter spp., Bacillus subtilis, B. licheniformis, B. megaterium, B. cereus, B. polymyxa, Aspergillus simplex, A. oryzae, A. niger, Trichoderma reesei, Saccharomyces spp., Pseudomonas, Mycobacteria, Achromobacter, Geotrichum marinum, Thirumalachar sp*. nov., or other hydrocarbon degradation bacterium. The nucleus 11 is preferably encapsulated by walls 12. The nucleus 11 may contain sufficient nutrients to sustain the microorganism; however, the walls 12 separate the nucleus from a source of bacterium nutrients (B) 13 which facilitate growth and are presented to the bacterium in the presence of an aqueous solution which penetrates or dissolves the wall 12. As illustrated in Fig. 16.2, these and similar cells 10 may be enclosed in envelopes 14 which have a time released coating 15, such as hydrophilic methacrylate or gelatin to regulate the time of contact with the environment once exposed to water, and these coatings may be of varying thickness or density, which not only varies the time of release but also varies the weight of the envelope.

Working of the Technology: When a breach of the hold or vessel containing the oil product occurs, the leaking of oil product through the breach in the vessel carries with it the pods, which when subject to water, become activated. The enteric coating may be removed by the water or by time, and the microorganism will be induced

to consume the escaped petroleum product, and such consumption may be enhanced by the nutrients packed with microorganisms, or these materials may be used primarily to keep the microorganisms hearty over time before they are activated by the presence of water. A Venturi-like effect carries the pods into the spill, dispersing the microorganism pods throughout the escaped oil product floating and submerged in contaminated water.

In summary, this invention teaches the following: first, a methodology of preemption through the introduction of remediating agents into the petroleum product before a spill occurs; second, time or controlled release remediating agents encapsulated or pelletized as products of varying densities to provide for relatively even dispersal throughout the petroleum product; third, remediating agents which may include magnetic or similar materials, or means permitting the removal of unused remediating agents from a body of petroleum product which has not been subjected to aqueous environment; fourth, including anaerobes for the metabolism of submerged petroleum products (which disperse throughout the petroleum bulk to exponentially increase the overall rate of bioremediation); and fifth, time release of aqueous components within the product to affect remediation of spills into nonaqueous environments.

Strategy 2

Combating Oil Slicks Floating on a Water Surface Using Natural Mineral Raw Materials Which Break Oil into Microscopically Small Parts: The second technology belongs to Swisstech Holding AG, Zug, CH. Swisstech Holding AG is a leading cleantech engineering group in onshore and offshore oil decontamination. The group is a worldwide pioneer in natural bioremediation products for oil-polluted substances. This group has offered highly efficient, economical, and ecological solutions. This group has filed a US patent application US20090120872A1 for this technology.

The inventor Kroh (2008), described a methodology for combating oil slicks floating on a water surface. The oil is broken down into microscopically small parts by way of the new composition. The new composition and the new method permit an in situ bioremediation. This strategy enables decomposition of the crude oil by accelerating the biological decomposition on location.

A composition according to the invention is a product that can be efficiently applied for the complete removal of spilled crude oil, also in port facilities or for tank ship accidents. The market-ready product is a dry, free-flowing mixture of solids, comprising a composition of natural mineral raw materials, naturally occurring as solids. These raw materials are obtained from primary or secondary deposits (quarries, stone pits, or other mineral deposits). They may also be recycled from certified non-contaminated natural mineral raw materials. Preferably the natural mineral raw materials are not thermally or chemically pretreated or treated.

The natural mineral raw materials, as a single compound, as mixtures, and in the final composition according to the invention, are chemically inert under natural surrounding conditions (on land, in the water, on ice). During long-time storage, transport, application, and also disposal, they are thus completely harmless for the people, animals, plants, and environment.

For the manufacture of the compositions according to the invention, the natural mineral raw materials are solely broken down, milled, and grinded to the necessary particle size, efficiently working from the invention. It results in a total particle surface of the compositions to which the polluting hydrocarbons, particularly crude oil, may adhesively attach in a quantitatively optimal manner (monomolecular level).

For combating an oil slick floating on a water surface, it is sufficient to scatter or blow the composition according to the invention over the oil slick. The composition according to the invention specifically has the advantageous and amazing characteristic that it spontaneously combines with the oil, and the sorbent without the formation of larger lumps sinks from the surface of the water to the bed of the water and settles there as a fine sediment. With the application of

adequate quantities of the composition according to the invention, one may ascertain hardly any toxic hydrocarbons at all in the treated water within a few hours or days. Laboratory trials have even shown that one may even achieve drinking water quality.

The oil is broken down into microscopically small parts by way of the new composition according to the invention. The powder particles bind the oil particles by adsorption, by way of arranging themselves around the oil droplet. Due to the higher specific weight of the powder according to the invention which is preferably between 2.5 and 3.5, preferably about 3.0, the parts of the oil-powder complex completely sink to the bed of the respective water. The present invention utilizes the physical sorption, a special form of adsorption, with which the sorptive is bonded by way of physical forces and not the formation of chemical bonding to the sorbent. The physical forces acting here, as a rule, are not directed and have the advantage that the bondings are reversible. The sorptive may thus be released from the sorbent again, which encourages the microbial breakdown of the oil.

The sunk oil may also neither be separated from the composition according to the invention by way of violent turbulence and thus may no longer drift to the surface of the water.

Since the crude oil is adsorbed onto the particles of the composition, the effective surface which is available for attack by the microorganisms breaking down the oil is increased enormously. By way of this the breakdown of the oil is rendered possible and is accelerated enormously until a complete decomposition of the oil. The microscopically small droplets serve as a substrate for the profuse development of the microorganisms taking part in the breakdown. The oil is broken down without any residue within 3–4 months, depending on the geological region and the prevailing environmental conditions. The biodegradation or the remediation therefore preferably takes place in situ. The difficult and cost-intensive suctioning of the oil-binding agent mixtures from the surface of the water and the subsequent disposal, dumping, and reprocessing of this highly problematic waste, which as mentioned above often leads to the fact that no measures at all are taken for cleaning oil contaminations, are rendered superfluous by way of the inventive in situ decomposition of the toxic hydrocarbon compound.

Microorganisms which are suitable for the decomposition of crude oil or other hydrocarbon contamination are added to the composition and form an important constituent of the product in one advantageous embodiment. The microorganisms are formulated such that its storage capability and flowing ability are ensured. The formulation is preferably effected in the form of pellets or capsules. In particular, national or local regulations are to be observed and adhered to with the application of nonindigenous microorganisms. Since these may be admixed without any problem into the suitable flowable formulation, even on location, the microorganism constituent may be adapted to the regulations which apply to the respective location of application without significant effort. A new solution for the previously mentioned problem and the task, which is derived from it, is specified by the present invention as is characterized in the patent claims.

Working of the Technology: So much agent according to the invention in the composition mentioned above was scattered onto the oil, floating essentially on the surface of the saltwater (a part of the oil however was also distributed in the water in the form of small droplets) that the complete oil layer floating on the water surface was covered with a roughly uniformly thin layer of the agent according to the invention.

Already a few seconds after scattering an agent according to the invention with 40% silica sand (grain size 0.2–0.3 mm), 40% granite meal, 15% meal of siliceous limestone, and 5% $CaCO_3$, the agent bonded to the oil, disappeared from the surface of the water, and as a result slowly fell to the bottom of the test vessel as a fine sediment. The water became rapidly clearer, and oil could no longer be ascertained in the water optically as well as by smell, already after about 1 day. The oil was completely bonded in the sediment.

Strategy 3

Substrate Bioavailability Enhancing Solution: The third strategy for bioremediation has been developed by Kaiser and Collins (1995) and is protected by a US patent (US5561059A). The technology is about a chemical mixture which enhances the substrate bioavailability.

The invention describes a liquid substrate bioavailability enhancing solution that cofunctions as an emulsifier and cleaner for oil-contaminated surfaces and subsurfaces and as an enhancing nutritional liquid carrier for specially selected naturally occurring microbes to be added and indigenous organisms. The added specially selected microbes multiply in the host liquid. After application to oil-contaminated surfaces or subsurfaces as the liquid emulsifies the contaminants, the microbes efficiently begin the digestion of the microscopic particles produced thereby.

The method developed by Conard and Collins is comprised of two separate parts: the microbe enhancing emulsifier and cleaner and the selected naturally occurring microbes. The liquid part of the invention is an aqueous mixture of nonionic and anionic surfactants and other chemicals.

The method claimed by Conard and Collins has the following exclusive features:

- Provides environmental workers with a substrate bioavailability enhancing emulsifier and cleaner for control of hydrocarbon contamination
- Provides an efficient substrate bioavailability enhancing oil emulsifier and cleaner-carrier for the practical delivery of selected bioremediating microbes
- Provides substrate bioavailability enhancing emulsifier-cleaner that, when applied to oil-contaminated surface or substrate, is not detrimental to the indigenous microbial population
- Provides a liquid carrier that acts as a life-enhancing medium for the selected naturally occurring microbes that are added prior to application to an oil-contaminated surface or substrate biocatalyst
- Provides a liquid carrier that acts as a life-enhancing medium for the indigenous microbes that are present prior to application to an oil-contaminated surface or substrate
- Provides a carrier liquid whose components can also act as a food source for the naturally occurring microbes prior to application to an oil-contaminated surface of substrate
- Provides a carrier that stimulates the dormant naturally occurring microbes when added and provides for their rapid multiplication through the presence of an atomically bonded stable nonoxidizing oxygen biocatalyst added prior to application to the contaminates
- Provides a substrate bioavailability enhancing chemical mixture that may be diluted with water, thus providing an economical method for delivery to, and application upon, contaminated areas
- Provides a carrier for microbes that is not harmful to the intact normal human skin so that it can be safely handled by the applicators
- Provides a method of practical bioremediation by using emulsifiers that efficiently portion food for the microbes
- Provides a method of hydrocarbon cleanup that does not require transportation and relocation of the contaminants which contributes to further pollution of the environment
- Provides a carrier whose active components do not react with each other

These and other attributes of the invention are realized by combining into an aqueous base, emulsifiers, surfactants, and other ingredients such as the emulsifier nonylphenol ethoxylate, a surfactant such as linear alkyl aryl sodium sulfonate, a sequestering agent such as ethylenedinitrilotetraacetic acid – tetrasodium salt – an emulsifying solvent such as dipropylene glycol monomethyl ether, a pH control agent such as sodium silicate, and an atomically bonded oxygen source as part of the water component which serves as a nonoxidizing oxygen biocatalyst.

Working of the Technology: Selected live naturally occurring microbes are added to the liquid emulsifier at the job site prior to use. The microbes may be anaerobic microbes, aerobic microbes, or facultative microbes which are added under anaerobic or aerobic conditions. The microbes may be dormant live naturally occurring microbes packaged in powder form.

The liquid substrate bioavailability enhancing emulsifier of the present invention, when the dormant dry powder microbes are added, acts as a host liquid for the microbes and with the action of the biocatalyst causes the microbes to be activated and nourished to activate their reproduction cycle.

The combination of ingredients stimulates a very rapid arousal of the microbes from their dormant state, activating their rapid division so that when the liquid mixture is applied, the microbes are vigorous and reproducing exponentially.

The substrate bioavailability enhancing chemical mixture is applied to oil-contaminated surfaces, and upon contact with the hydrocarbons, the mixture of ingredients rapidly emulsifies the oil contaminates into tiny droplets. Just as milk fat (oil) is in a near colloid state in milk and thus does not easily rise to the surface as cream, the emulsifiers decrease the size of the contaminating oil and hold it in suspension because of the tiny droplet sizes. This exposes tremendous surface area to the activated oil-consuming microbes which can then efficiently begin bioremediation. Microbes can most efficiently biodegrade their food substrate if it is available in sizes smaller than themselves.

The encapsulation of the microscopic particles of oil in the resultant colloid state gives the microbes the important oil/water interface that is necessary, along with supplemental oxygen, for their efficient oil-digesting activities.

Strategy 4

Nutrient Material for Promoting Growth of Petroleum-Degrading Bacteria: The fourth strategy was devised by Felix and Hruza (1998) who are working for a US-based company named BioNutraTech, Inc. This company manufactures and distributes micro-encapsulated bio-nutrients that stimulate microbial activity for advanced commercial, industrial, and environmental bioremediation. Their proprietary products provide essential nutrients that stimulate and sustain the accelerated growth of highly desirable types of naturally occurring microbes that are already present in targeted wastes. The technology developed by BioNutraTech was first disclosed publically through a patent application WO-1999046210A1.

The method disclosed by Felix and Hruza uses a particulate material for promoting growth of petroleum-degrading bacteria. Each particle of the nutrient material comprises a core of water-soluble, microbial available nutrients selected from the group consisting of nitrogen in the form of ammonium or urea compounds, phosphorous in the form of microbial available phosphate compounds, iron in the form of microbial available iron compounds, and oxygen in the form of various hydrogen peroxide compounds. The coating formulation of this invention uses a mixture of saturated and unsaturated fatty acids to form a coating material which is readily biodegradable, has physical properties making it efficient for encapsulating microbial nutrients, increases the oil phase partitioning of the composite, and reduces the cost of manufacture. More particularly, the encapsulation for the core of nutrients is formed of an oleophilic and biodegradable coating comprising oleic acid and a carboxylic acid selected from the group consisting of stearic acid, palmitic acid, and mixtures thereof. The preferred ratio of oleic acid to the selected carboxylic acid is between about 70:30 and about 30:70 by weight.

The invention also provides a process of manufacturing the coated nutrient material (Fig. 16.3), which comprises mixing microbial nutrients together as dry ingredients and grinding them into a powder sufficient to pass through a number 40 sieves. A coating mixture is prepared from commercial grade stearic acid and oleic acid at a preferred ratio of 40:60 by weight.

The coating mixture is melted by heating to 100°C and resolidified to form a homogeneous composite and remelted at 100°C. Upon cooling to 65°C, the coating is blended into the nutrients at a ratio of 90% nutrients to 10% coating by weight.

Working of the Technology: Biodegradation potential was assessed by treatment of artificially weathered crude oil treated with an oleophilic nutrient mixture without an oxygen-releasing compound. An identical untreated control was maintained for comparison. The oil was then

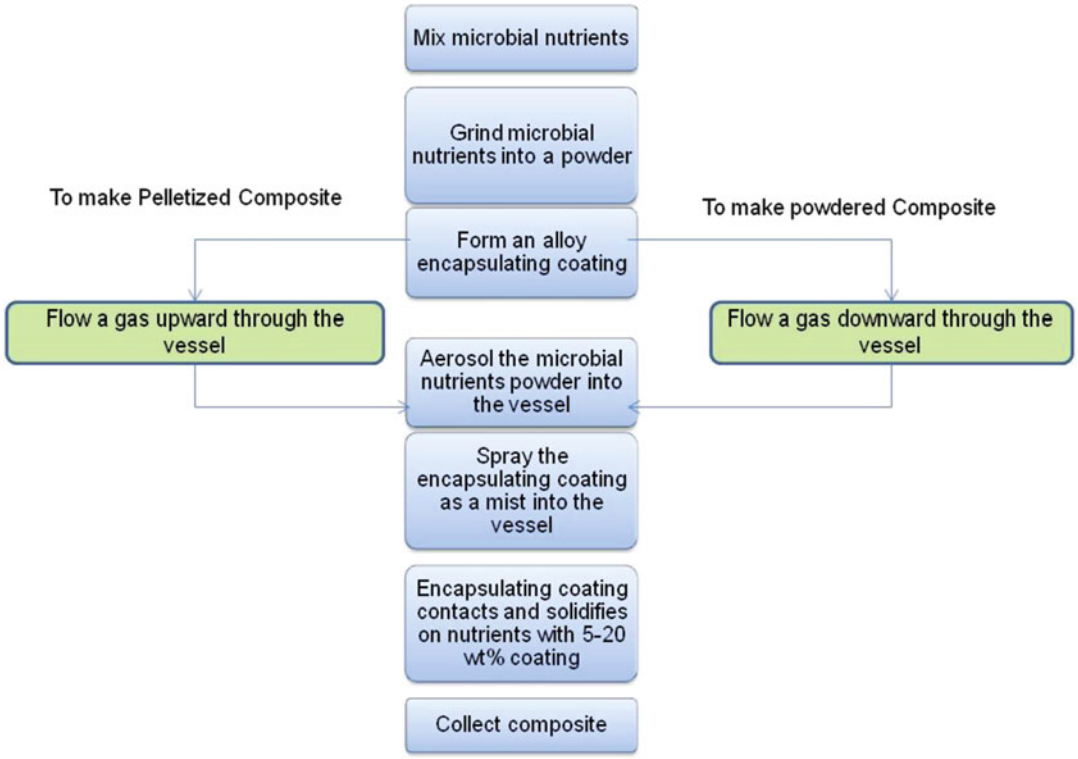

Fig. 16.3 Is a flow diagram of the method of manufacturing the present composite material

treated with 0.30 g of a nutrient mixture composed of 0.256 g $(NH_4)_2SO_4$, 0.011 g of KH_2PO_4, and 0.003 g of $FeSO_4\ 7H_2O$, coated with a mixture of 0.018 g oleic acid and 0.012 g of stearic acid. The reaction vessel was continuously aerated with bubblers at a rate of 250 mm of air per minute. After 48 h, a follow-up application was made using only one-half the amount of nutrient mixture as in the original application. Percent difference in reduction of oil between the treated and untreated oil was measured after each time period.

Strategy 5

Enriched Steady-State Microbial Consortiums for Microbial Enhanced Oil Recovery and In Situ Biore-mediation: The fifth strategy for bioremediation comes from a team of scientists working for a US-based company DuPont. The technology disclosed by Hendrickson et al. (2010) of DuPont through a patent application number WO2010096537A1 uses enriched steady-state microbial consortiums for microbial enhanced oil recovery, and in situ bioremediation of hydrocarbon-contaminated sites, under anaerobic denitrifying conditions, is disclosed. The consortium is identified by obtaining environmental samples comprising indigenous microbial populations exposed to crude oil and enriching the said populations per enrichment protocol. The enrichment protocol employs a chemostat bioreactor to provide a steady-state population. The steady-state population may be characterized by using phylogenetic DNA sequence analysis techniques, which include 16S rDNA profiling and/or DGGE fingerprint profiling as described herein. The steady-state population is further characterized as an enriched consortium comprising microbial constituents having relevant functionalities for improving oil recovery or in situ bioremediation of hydrocarbon-contaminated environmental sites. The steady-state enriched consortium may

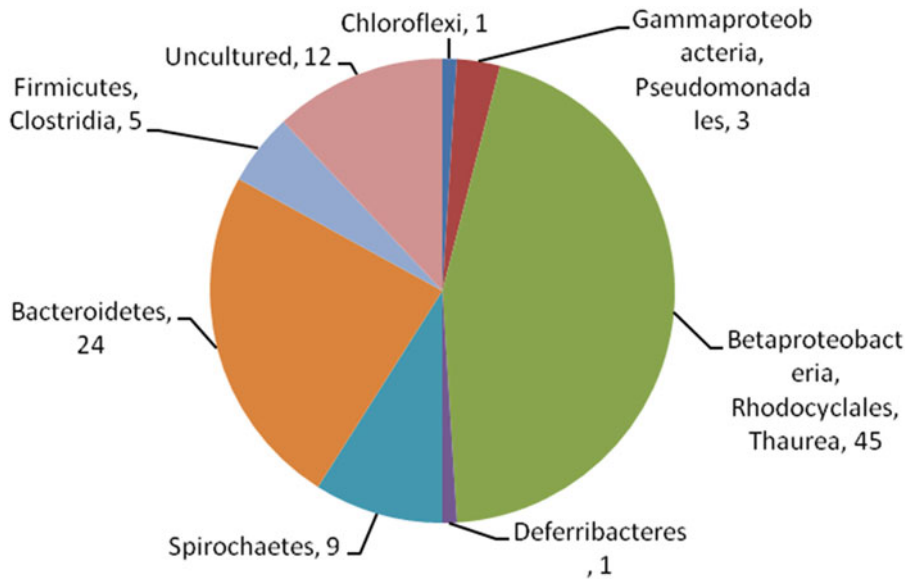

Fig. 16.4 Distribution of microorganisms in the parent POG1 consortium after 3 months in second-generation parent populations as determined by 16S rDNA identities

grow in situ, under reservoir conditions, using one or more electron acceptors and the reservoir's crude oil as the carbon source for microbial enhancement of oil recovery or in situ bioremediation of hydrocarbon-contaminated environmental sites. The steady-state consortium may be used with other microorganisms to enhance oil recovery in reservoirs or wells or in situ bioremediation of hydrocarbon-contaminated environmental sites with analogous reservoir conditions of the selected/targeted wells.

The method described for in situ bioremediation of hydrocarbon-contaminated environmental sites comprises an isolated consortium of microbial species, comprising one *Thauera* strain and two other strains selected from the group consisting of *Azoarcus* sp. *Pseudomonas* sp. *Azotobacter* sp. *Bacteroides* sp. *Clostridium* sp. *Anaerovorax* sp. *Finegoldia* sp. *Spirochetes* sp. *Deferribacter* sp. *Flexistipes* sp. *Chloroflexi* sp. *and Ochrobactrum* sp. (Fig. 16.4).

Working of the Technology: Enrichment of a microbial consortium on targeted oil as the carbon source under denitrifying anaerobic condition is carried out. Parent enrichment cultures and a screening protocol were developed to identify microbes capable of growth under anoxic conditions on either crude oil or its components or samples from a hydrocarbon-contaminated site as the sole source of carbon. These soil suspensions were used as an inoculum into 60-mL serum vials that contained 2:1 v/v of the minimal salts medium (20 mL) and the autoclaved crude oil (10 mL).

The parent POG1 consortium cultures were examined for their ability to release oil from sand in a visual oil release assay using the microsand column described above. Inocula from early parallel enrichment cultures of the second-generation parent POG1 consortium and one third-generation culture (EH40:1) with high nitrate concentration (−1,600 ppm) were tested in this assay. All enrichment cultures were grown anaerobically in the SL10 minimal salts medium using ACO oil as the carbon source and nitrate as the electron acceptor until turbidity was observed. A 4.0 mL aliquot of each inoculum was added to the 13-mm glass tubes either directly or diluted 1:2 with the minimal salts medium. The micro-

sand columns were placed in each glass tube, immersed in the medium/cell inoculum with the tapered neck of the Pasteur pipettes pointing up. The outer vials were sealed in the anaerobic Coy chamber and allowed to incubate at ambient temperatures for the next several weeks. Each column was periodically checked for oil release. Cultures that enhanced release of oil over background (sterile medium) were presumed to have altered the interaction of the oil with the sand surface. Oil released from the sand was visualized by the released oil collecting in the tapered neck of the Pasteur pipettes or forming droplets on the surface of the sand layer. Oil release was observed for some of the POG1 parent enrichment cultures as rapidly as only 3 h after inoculation.

Strategy 6

Microemulsion as a Biodegradation Accelerator: The sixth technology is assigned to Elf Aquitaine and is protected as US patent US6190646B1. Elf Aquitaine was a French oil company which merged with Total Fina to form Total Fina Elf. The new company changed its name to Total in 2003. The technology is about a nutrient microemulsion in spray form, useful as a biodegradation accelerator. This technology was invented by Tellier et al. (1999).

The invention describes a microemulsion which is useful as a biodegradation accelerator which is nontoxic, which is stable at temperatures from −10 to +50°C, and which has fluidity at +5°C which is sufficient for use in the applications desired.

This microemulsion, which is useful as a biodegradation accelerator, contains at least one nitrogenous compound such as amino acids, proteins, urea, and its derivatives from 10 to 35% by weight of a phosphorus surfactant compound of the alkyl or alkenyl phosphoric ester type, the ester being a monoalkyl- and/or dialkyl- or monoalkenyl- and/or dialkenylphosphoric ester from 3 to 20% by weight of at least one cosurfactant, a compound selected from the group consisting of vegetable oils, animal oils and fatty acids, and a plasticizer, said emulsion having a viscosity at 5°C less than or equal to 200 mPa sec and stable at a temperature of −10 to +50°C.

In the present invention, it is the combined effect of the surfactants derived from the above specific phosphoric esters, with a cosurfactant or a mixture of cosurfactants, which assures the stability of the microemulsion down to a very low temperature, at least −10°C, and allows the product to be used without difficulty in any cold environment.

Working of the Technology: There is an accelerator effect on the biodegradability of microemulsions of the invention, with regard to hydrocarbons with a radiorespirometry pilot. The measurement is conducted with the aid of a laboratory apparatus called a radiorespirometer. It enables continuous observation of the digestion of radioactive hydrocarbons while evaluating the abiotic losses on each culture which avoids incubating with sterile cultures.

Hundred milliliters of a reaction medium is formulated from synthetic seawater, bacterial flora, and phenanthrene labelled with ^{14}C, biodegradable with difficulty, at 100 mg/L, in culture flasks or 250-mL Erlenmeyer flasks equipped with two lateral Torion (joints), with one being connected to the inlet for oxygen, and the other being connected to a trap for hydrocarbons formed by ORBO-43 columns sold by Supelco. The flow of oxygen for aeration is adjusted to 5 mL/min, and the medium is agitated with a back-and-forth agitator at 80 oscillations per minute. These reactors, thus agitated, are placed in the dark to incubate at 20°C for 1 month. Evaporated hydrocarbons are absorbed on the hydrocarbon traps. The flow of air entrains CO_2 labelled with ^{14}C derived from digestion of the phenanthrene into a CO_2 trap formed by a solution of 4N sodium hydroxide.

During this incubation for 1 month, in order to measure the kinetics of digestion, samples are taken every 2 or 3 days, depending on the results obtained. Three aliquot samples of 0.5 mL of the solution of 4 N sodium hydroxide are placed for measurement in 10 mL of Hionic-Fluor cocktail from Packard.

The bacterial flora used to test the effectiveness of the microemulsions, or rather of the surfactant/cosurfactant pair, is a complex natural flora of

marine origin. Before starting the tests, this flora is reactivated by being kept at 80°C on rich medium Marine Agar 2216 from Difco for 8 h. Then it is precultured on synthetic seawater of the Instant Ocean type at 33 g/L and enriched with 50 mg/L of light crude Arab oil for 2 days in the presence of nitrogenous and phosphorous nutrients, ammonium chloride, and potassium hydrogen phosphate, in concentrations such that the ratios C/N/P are equal to 106/16/1.

The labelled phenanthrene used is $^{9-14}$C, Sigma product 31,528-1. The tests are carried out in nine reactors placed in series, all containing the same reaction medium formed from synthetic seawater, labelled phenanthrene at 100 g/L, activated bacterial flora at 10% v/v, and a neutral pH buffer, TRIS at 6 g/L, these concentrations being reported as the reaction medium. Various microemulsions of the invention and of the prior art are introduced into 8 of these reactors. The concentrations of the microemulsions in the reaction media at the beginning of the trials are 10 mg/L.

A better digestion of the phenanthrene is noted in the presence of microemulsions × 16 and × 20. For certain other formulations of the invention, a certain latent period is observed before digestion is produced.

Strategy 7

Nutrient Concentrate for Accelerated Growth of Hydrocarbon-Consuming Microorganisms: The seventh representative technology invented by Holtwiesche, Bettina; Weiss, Albrecht; and Boehme, Adelheid, is assigned to Henkel and is protected by a US patent number US5635392A. Founded in 1876, Henkel holds globally leading market positions both in the consumer and industrial businesses. Headquartered in Düsseldorf, Germany, it has about 48,000 employees worldwide. The present invention provides a nutrient mixture which has an emulsifying effect and would contain P- and N-yielding substances, but would be made up of very few pure C-compounds.

This nutrient mixture leaves very few residues behind in the soil sample to be treated. The described nutrient mixture would prefer the pollutant-degrading microorganisms to other microorganisms.

This invention relates to a nutrient concentrate for stimulation and as a growth aid for the accelerated growth of hydrocarbon-consuming microorganisms for their use in the biological degradation of organic components, containing water-soluble and/or oil-soluble compounds of Phosphorus (P) and Nitrogen (N) present in admixture with other water-soluble and/or oil-soluble organic mixture components which are at least partly endowed with nutrient character for the growth of the microorganisms, characterized in that it is in the form of a liquid water-based preparation and contains one or more esters of phosphoric acid as emulsifier and P-source and, if desired, one or more water-soluble or water-dispersible N-sources.

The present invention also relates to the use of the nutrient mixtures in combination with concentrates of microorganisms which are capable of degrading hydrocarbon compounds and which are preferably of natural origin. Important applications include the remediation of soils, freshwater, and/or saltwater through the elimination of pollution based on hydrocarbon compounds and also the treatment of working equipment, pipes, and large vessels, including tankers and the like, using the nutrient mixtures according to the invention.

In one particular embodiment, the invention relates to the use of the nutrient mixtures, more particularly in combination with microorganism concentrates capable of degrading hydrocarbon compounds, for the elimination of oil-wetted cuttings from geological land-supported or offshore drilling, for example, from the development of geological occurrences.

In the context of the invention, particular significance is attributed to the phosphoric acid esters used. These compounds act on the one hand as a P-source and on the other hand as emulsifiers. Although the way in which they act is not fully understood, it may be assumed that the compounds collect at the interface between the hydrophobic pollutant and the water present in the soil sample and, on the one hand, develop an emulsifying effect and, on the other hand, keep the phosphorus source available in the immediate vicinity of the pollutant so that it is useful in par-

ticular to those microorganisms which are capable of degrading the pollutants. Preferred phosphoric acid esters are those which can be taken up by microorganisms. Accordingly, amongst the compounds which meet this requirement, particular significance is attributed to the phospholipids. Phospholipids are amphiphilic substances which are obtained from vegetable or animal cells. Preferred phospholipids are the glycerophospholipids which, normally, are also known as lecithin. The sphingophospholipids are less preferred. Known compounds which may be used in accordance with the invention are the diacyl phospholipids, phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols, phosphatidylserines, phosphatidylglycerols, phosphatidylglycerol phosphates, diphosphatidylglycerol, *N*-acyl-phosphatidylethanolamine, and phosphatidic acid. Preferred compounds are the monoacyl phospholipids, lysophosphatidylcholines, lysophosphatidyl ethanolamines, lysophosphatidyl inositols, lysophosphatidyl serines, lysophosphatidyl glycerols, lysophosphatidyl glycerophosphates, lysodiphosphatidyl glycerols, lyso-*N*-acylphosphatidylethanolamines, and lysophosphatidic acid. By virtue of their accessibility, the expert will mainly use commercially available phosphatidyl glycerides which are marketed as vegetable or animal lecithins and cephalins. These preparations are generally obtained from oils, such as wheat germ oil or cottonseed oil or soybean oil. According to this strategy, preference is attributed to the enzymatically hydrolyzed glycerophospholipids (enzymatically hydrolyzed lecithin), which are more hydrophilic in character through the elimination of a fatty acid residue. The only exceptions are those products which have lost their phosphoric acid component as a result of the enzymatic hydrolysis.

In addition to or instead of the phospholipids mentioned above, partial esters of phosphoric acid with fatty alcohols, more particularly corresponding partial esters with linear fatty alcohols, may also be used as the P-source of the nutrient concentrates according to the invention. Esters of relatively short-chain fatty alcohols, for example, C_{6-10} fatty alcohols, are particularly suitable. However, alkyl phosphates with relatively long-chain fatty alcohols, that is, C_{12-24} fatty alcohols, may also be used. Fatty alcohol ether phosphates are also suitable. These are phosphoric acid partial esters of ethoxylated fatty alcohols, the fatty alcohols containing 8–24 carbon atoms and being ethoxylated with 1–10 mol and preferably 4–6 mol of ethylene oxide per mole of fatty alcohol.

The phosphoric acid esters mentioned are used in the nutrient concentrates according to the invention in quantities of 10–40% by weight and preferably in quantities of 20–30% by weight.

In addition, the nutrient concentrates according to the invention contain an N-source in the form of inorganically and/or organically bound nitrogen. N-sources which only contain organically bound nitrogen are preferred because the use of inorganically bound nitrogen can result in salinization of the soil.

Working of the Technology: A nutrient concentrate was prepared from glycerophospholipid (enzymatically hydrolyzed lecithin, trade name: Lipotin® NE, a product of Lucas Meyer) 20% by weight, urea 20% by weight, alkyl polyglycoside (C chain length: C.sub.8 to C.sub.16, D.P.: approx. 1.4) 2% by weight, and water ad 100% by weight. Substrate: 2kg of sand polluted with 7,000 ppm of crude oil. Incubation temperature: 16°C; observation period: 42 days; treatment interval: 0.4 g nutrient concentrate/kg sand every 10 days; pollutant analysis method: DIN 38409H18. The pollutant analysis method according to DIN 38409H18 comprises determining the specific spectral absorption of hydrocarbon mixtures by infrared spectroscopy in an organic solvent extract. The number of 'degradation specialists', that is, the microorganisms capable of degrading the contamination, was determined by initially determining the total number of growing microorganisms or microorganisms capable of growth and then determining the number of degradation specialists from the result obtained by transferring the cultures to a nutrient plate containing hexadecane as sole C-source. It was undoubtedly proved that the nutrient concentrate activates the degradation potential of marine flora.

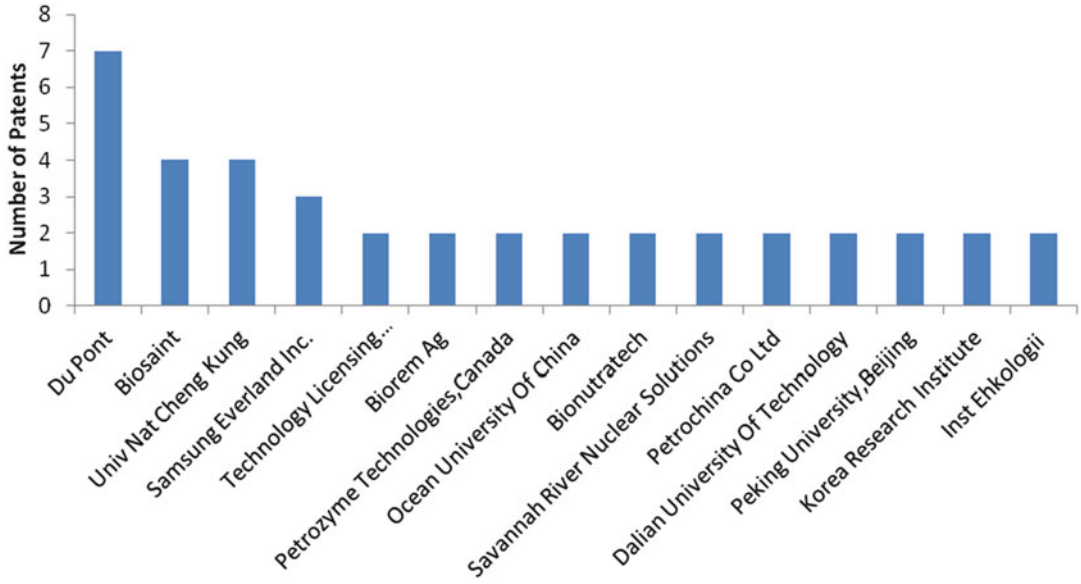

Fig. 16.5 Number of patent families filed by different assignees

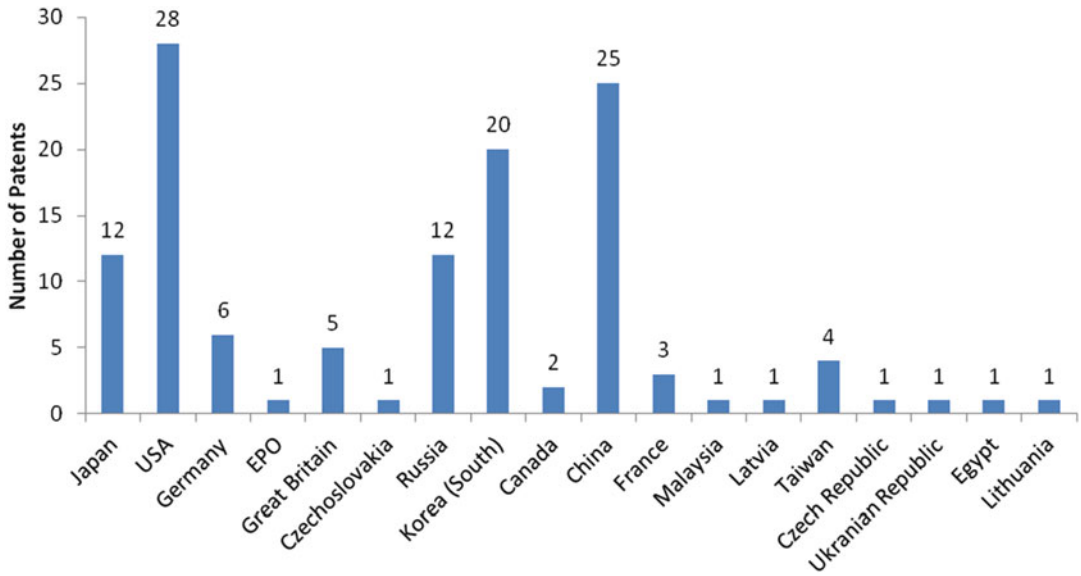

Fig. 16.6 Number of patent families filed for microorganism-based bioremediation technologies

Top Assignees and Countries in the Domain of Bioremediation

Figure 16.5 shows that US-based company DuPont is the most aggressive company working towards developing microorganism-based technologies for bioremediation of oil-contaminated sites followed by Biosaint; Univ Nat Cheng Kung; Samsung Everland Inc.; Technology Licensing Organization, Japan; Biorem AG; Petrozyme Technologies, Canada; Ocean University of China; and BioNutraTech.

The USA is on the top of all the countries in patenting activity (Fig. 16.6). US-based companies E.I. du Pont de Nemours and Company, Wilmington; BioNutraTech Inc., USA, and Savannah River Nuclear Solutions LLC are actively working in this direction. In China the universities are very actively working in development of bioremediation technologies, prominent amongst these are Ocean University of China, Dalian University of Technology, and Peking University, Beijing, and in companies, PetroChina Co. Ltd. is very active. Korea is an East Asian country that is very active after China; main companies in Korea are Biosaint Co. Ltd., Samsung Everland Inc., and Korea Research Institute of Bioscience & Biotechnology. In Japan, Taisei Corp., Tosoh Corp., and Fukushima University are the top inventing companies.

References

Atlas RM, Hazen TC (2011) Oil biodegradation and bioremediation: a tale of the two worst spills in U.S. history. Environ Sci Technol 45:6709–6715

Chamkha M, Mnif S, Trabelsi Y, Sayadi S (2011) Isolation and characterization of Klebsiella oxytoca strain degrading crude oil from a Tunisian offshore oil field. J Basic Microbiol 51(6):580–589

Chandran P, Das N (2011) Degradation of diesel oil by immobilized *Candida tropicalis* and biofilm formed on gravels. Biodegradation 22:1181–1189

Churchill PR, Dudley RJ, Churchill SA (1995) Surfactant enhanced bioremediation. Waste Manag 15:371–377

Felix E, Hruza SL (1998) Method and composition for enhanced bioremediation: WO1999046210A1, Bionutratech Inc., US

Galindo H, Revah S, Cervantes FJ, Arriaga S (2011) Effect of surfactant and oil additions in the biodegradation of hexane and toluene vapours in batch tests. Environ Technol 32:167–173

Hendrickson ER, Luckring AK, Keeler SJ, Perry MP, Choban ER (2010) A steady state anaerobic denitrifying consortium for application in In-Situ bioremediation of hydrocarbon-contaminated sites and enhanced oil recovery, Publication number: WO2010096537A1, E. I. Du Pont De Nemours And Company, Wilmington

Kaiser CE, Collins JR (1995) Substrate bioavailability enhancing chemical mixture for use in bioremediation, Publication number: US5561059A

Kirschner L (1999) Product and method for pre-emptive potential in remediation of oil spills, Publication number: EP1088109B1

Korotkevych O, Josefiova J, Praveckova M, Cajthaml T, Stavelova M, Brennerova MV (2011) Functional adaptation of microbial communities from jet fuel-contaminated soil under bioremediation treatment: simulation of pollutant rebound. FEMS Microbiol Ecol 78:137–149

Kroh W (2008) Composition and bioremediation method for water polluted by hydrocarbons, Publication number: US20090120872A1, Swisstech Holding Ag, Zug, Ch

Lin TC, Pan PT, Young CC, Chang JS, Chang TC, Cheng SS (2011) Evaluation of the optimal strategy for ex situ bioremediation of diesel oil-contaminated soil. Environ Sci Pollut Res Int 18:1487–1496

Liu X, Wang Z, Zhang X, Wang J, Xu G, Cao Z, Zhong C, Su P (2011) Degradation of diesel-originated pollutants in wetlands by Scirpus triqueter and microorganisms. Ecotoxicol Environ Saf 74:1967–1972

Lloyd-Jones G, Laurie AD, Hunter DWF, Fraser R (1999) Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. FEMS Microbiol Ecol 29:69–79

MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang Y-J, White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 65:3566–3574

Mesarch MB, Nakatsu CH, Nies L (2000) Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. Appl Environ Microbiol 66:678–683

Mohite BV, Pawar SP, Morankar A (2011) Isolation, selection and biodegradation profile of phenol degrading bacteria from oil contaminated soil. Bull Environ Contam Toxicol 87:143–146

Tellier J, Basseres A, Brochette P, Espert A (1999) Nutrient microemulsion in spray form, useful as a biodegradation accelerator, Publication number: US6190646B1, ELF Aquitaine, Courbevoie, FR

Tiehm A (1994) Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl Environ Microbiol 60:258–263

Zhang W, Li J, Huang G, Song W, Huang Y (2011) An experimental study on the bio-surfactant-assisted remediation of crude oil and salt contaminated soils. J Environ Sci Heal A Toxic Hazard Substrate Environ Eng 46:306–313